The effect of antibiotic treatment on *Haemophilus parasuis* colonization, disease and immunity

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DEDICATION

To my husband, Arnaldo, and my son, Benjamin.

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GENERAL INTRODUCTION

Bacterial diseases cause dysfunction, pain and even death in pigs of all ages. The use of antimicrobial agents is one of the most cost-effective tools in the efficient production of pork by reducing the distress and speeding the recovery in infected animals (Friendship, 2000). Antimicrobials are widely used in swine especially during treatment of respiratory and enteric diseases (De Briyne et al., 2014). In addition to treatment of sick animals, antimicrobials are used for control and prevention in healthy animals, specifically when periodic outbreaks of bacterial infections happen and the probability that most animals become infected is high (Barton, 2014). However, the recent emergence of antibiotic resistant bacteria in humans is putting into question the antibiotic practices in food animals. (Aarestrup et al., 2008; Wallinga and Bursch, 2013; Anonymous, 2015). Therefore, there is a need to promote responsible use of antimicrobials in swine.

Glasser's disease is an important source of economic losses in current swine production systems. Glasser's disease is caused by *Haemophilus parasuis,* a Gram-negative bacterium that affects pigs, and is characterized by polyserositis, arthritis, meningitis, pneumonia and sudden death. *H. parasuis* also colonizes the upper respiratory tract (URT) of healthy pigs and colonization may be an important event in the development of immunity. For instance, inoculation of young piglets with pathogenic *H. parasuis* has been shown to reduce nursery mortality (Oliveira et al., 2001a; 2003a). It was hypothesized that a protective immune response to *H. parasuis* was activated when pathogenic *H. parasuis* strains colonize the URT of pigs (Pijoan et al., 1997). Therefore, factors that can disrupt *H. parasuis* colonization, such as antimicrobial treatment, could affect the pig's ability to develop a protective immune response against Glasser's disease.

Antimicrobials have been widely used to treat and control Glasser's disease (Aragon et al., 2012). Antimicrobials have also been shown to reduce *H. parasuis* colonization in weaned pigs (Vilalta et al., 2012) and anecdotal field observations suggest that mass treatment with antibiotics in pigs at weaning may render them more susceptible to Glasser's disease later on. One hypothesis that would explain this observation is that early elimination (or lack of colonization) of *H. parasuis* may interfere with the development of immune responses. This mechanism has been previously demonstrated for other pathogens (North, et al., 1981; Su et al., 1999; Griffin et al., 2009; Sjolund et al., 1009). Therefore, evaluating the impact of antimicrobial treatment on immune responses to *H. parasuis* should enable us to develop better treatment programs that will prevent reinfections. Towards this end, the goal of this PhD dissertation is to investigate the influence of antimicrobial treatment on *H. parasuis* colonization and infection, and its effect on the development of immune responses against *H. parasuis* in swine. The central hypothesis of this Ph.D. dissertation is that antimicrobial interfere with *H. parasuis* colonization and infection and, as a result, they affect the protective immune responses against Glasser's disease.

Little is known about how the pig immune system responds to *H. parasuis* colonization and infection. A serological test is needed to study the immune response to *H. parasuis*. However, most attempts to measure pig immune responses to *H. parasuis* used whole cell-based serological assays to measure serum IgG responses (Martin de la Fuente et al., 2009a; Cerda-Cuellar et al., 2010). Such serological methods lack sensitivity (do not detect responses against all *H. parasuis* strains) and specificity (may cross-react with responses against other Gram-negative bacteria). Therefore, a consistent and accurate serological test to characterize the development

of specific antibodies against *H. parasuis* in pigs is lacking. The first research chapter (chapter 2) describes the identification of a novel immunogenic and species-specific protein in *H. parasuis*, the oligopeptide permease A (OppA) protein. OppA is a transmembrane protein in Gram-negative bacteria responsible for capturing substrates from the environment (Monet, 2003). In chapter 2, the OppA protein is used to develop a serological test that detects antibodies against a wide range of *H. parasuis* strains. OppA immunogenicity was evidenced by the fact that only convalescent pigs or pigs vaccinated with recombinant OppA (rOppA) developed antibodies specifically against it.

Another tool that is needed to study the complex relationship between *H. parasuis* infection, immune response and antibiotics is an experimental model for *H. parasuis* colonization. URT colonization by *H. parasuis* is considered a first step for *H. parasuis* infection. Numerous experimental inoculation models have been described for *H. parasuis*. However, those models were mainly designed to reproduce Glasser's disease, and not *H. parasuis* colonization. In addition, they used colostrum deprived pigs, which do not fully represent the conventional pig in terms of the immune response they develop or the interactions that exist with the nasal microbiota. Therefore, there is a need for an infection model that simulates an asymptomatic colonization of the URT of pigs by pathogenic *H. parasuis* strains. In chapter 3, an experimental model that mimics nasal colonization in conventional pigs is proposed. We document that conventional pigs naturally carrying *H. parasuis* in their noses can be experimentally colonized by a pathogenic *H. parasuis* strain without developing Glasser's disease. Absence of disease was evidenced by absence of clinical signs, and lesions, and lack of *H. parasuis* isolation and *H. parasuis* DNA detection in blood and

systemic tissues. The development of a pig model that mimics upper respiratory tract colonization in conventional pigs is a valuable tool to study *H. parasuis* colonization and infection.

Alterations of *H. parasuis* URT colonization at a young age have been associated with occurrence of Glasser's disease. Antimicrobials are commonly used to treat respiratory diseases in pigs. Specifically, enrofloxacin is used to control Glasser's disease at weaning, a time when pigs are being exposed to different *H. parasuis* strains. We hypothesize that the administration of enrofloxacin can interfere with the colonization of the URT with *H. parasuis*. However, there is no information on the effect of enrofloxacin on *H. parasuis* colonization. Chapter 4 documents the reduction of both, the number of pigs positive to *H. parasuis* and the levels of *H. parasuis* in tonsils and nasal cavity of conventional pigs during the first week after treatment with enrofloxacin.

Antimicrobials help the immune system to fight off disease by rapidly reducing or eliminating the bacterial challenge. However, early antimicrobial treatment can also interfere with the ability to mount an effective immune response by eliminating the pathogen before the immune system is activated. Chapter 5 describes the pig immune responses to pathogenic *H. parasuis* when pigs are treated with enrofloxacin before or after *H. parasuis* inoculation, and evaluates subsequent effects on protection against reinfection. Increase of serum IgG antibodies was observed after *H. parasuis* inoculation only when pigs were treated with enrofloxacin before inoculation and not when pigs were treated after inoculation. Such seroconversion was associated with protection against reinfection, and the effect of antibiotic treatment on the

development of such protection depended on the timing of infection and antibiotic administration.

The final chapter (chapter 6) of this dissertation summarizes the findings and conclusions of this thesis, discusses limitations of the studies, and addresses future research needs.

CHAPTER 1

LITERATURE REVIEW

Sections of this chapter have been submitted to:

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1.1 *Haemophilus parasuis*

Haemophilus parasuis is a Gram-negative bacterium member of the family *Pasteurellaceae.* Bacteria from the family *Pasteurellaceae* infect humans and a broad range of animals, often with marked host specificity, and are the cause of various diseases with different pathological features. The family *Pasteurellaceae* includes the genera *Haemophilus, Pasteurella,* and *Actinobacillus*, with pathogenic and commensal bacteria among them (Nicolet, 1990). Moller and Kilian (1990) performed a systematic examination of the variety and ecology of members of the family *Pasteurellaceae* in the porcine upper respiratory tract (URT) demonstrating that healthy pigs of all ages harbor a wide spectrum of *Pasteurellaceae* species in their URT.

H. parasuis is one of the most important disease causing bacteria affecting pigs. *H. parasuis* causes the syndrome known as Glasser's Disease, which is characterized by polyserositis and arthritis. *H. parasuis* is considered to be present in all major swine-rearing countries and remains a significant disease in modern production systems. In the USA Glasser's disease is considered one of the main infectious problems in the nursery, but it can also affect growing pigs and sows (Aragon et al., 2012).

Several models and routes of infection have been tested to study virulence, pathogenesis and immunity to *H. parasuis* (Morozumi et al., 1981, 1982; Nielsen, 1993; Amano et al., 1994; Vahle et al., 1995; Oliveira et al., 2003a). Porcine polyserositis and arthritis due to *H. parasuis* infection are typically observed after weaning in affected herds (Oliveira et al., 2002). The clinical syndrome is typically acute with anorexia, nervous signs, and lameness with one or more joints affected.

Pigs often develop fever. Pigs that survive the acute disease have rough hair coats, poor weight gain and chronic lameness (Menard and Moore, 1990). Macroscopic lesions at necropsy reveal variable amounts of fibrinous to fibrinopurulent exudate present on multiple serosal surfaces including pleura, pericardium and peritoneum. Joints and meninges can also be affected with purulent arthritis and meningitis, respectively (Menard and Moore, 1990; Amano et al., 1994). Microscopically, these inflammatory lesions consist of deposition and infiltration of fibrin, neutrophils and occasionally macrophages (Hoefling, 1991; Vahle et al., 1995). Less commonly, *H. parasuis* infection can cause acute septicemia in which cyanosis, sub-cutaneous and pulmonary edema, and acute death can occur without the typical serosal inflammation (Peet et al., 1983). In the field, factors believed to be involved in Glasser's disease occurrence are stress related conditions, co-infection with viral pathogens, levels of maternal immunity, strain variability and differences in virulence (Aragon et al., 2012).

The existence of 15 distinct serovars was described by Kielstein and Rapp-Gabrielson (1992). They also demonstrated differences in virulence among the serovars after intraperitoneal inoculation of specific-pathogen-free (SPF) pigs. Studies also revealed the existence of high genetic heterogeneity among *H. parasuis* isolates, including genetic differences within serovar groups and among nontypeable isolates (Oliveira et al., 2003b; Olvera et al., 2006). Several studies indicate that *H. parasuis* strains differ in their ability to cause systemic disease in swine (Oliveira et al., 2003b; Olvera et al., 2006). Recently, the complete genome of a virulent Chinese *H. parasuis* isolate was sequenced (Yue et al., 2009). The genome size was about 2.3 Mb in a single circular chromosome composed of 2,292 predicted coding sequences. Several putative

virulence-associated genes were detected in the genome sequenced. Identification of novel putative *H. parasuis* virulence factors and mechanisms of virulence has been an active area of investigation (Zhou et al., 2009; Hong et al., 2011; Yuan et al., 2011; Fu et al., 2013). Some of the traditional virulence factors described for *H. parasuis* include fimbriae (Munch et al., 1992), capsule (Morozumi and Nicolet, 1986a), and lipooligosaccharide (LOS) (Biberstein, 1990).

Fimbriation has been described for *H. parasuis,* and fimbriae may play a role in the colonization of the porcine URT (Munch et al., 1992). *H. parasuis* colonizes the nasopharyngeal mucosal epithelium of pigs by attaching to the mucosal surface (Vahle et al., 1997). *H. parasuis* has also been consistently isolated from nasal cavity, tonsil, and trachea of pigs experimentally inoculated (Amano et al., 1994; Segales et al., 1997; Vahle et al., 1997; Kirkwood et al., 2001). For *H. parasuis,* the expression of fimbriae is dependent on cultivation and growth conditions. Fimbriae could not be identified when *H. parasuis* was grown using conventional cultivation methods, but rather when it was in contact with living tissue. Embryonated hen eggs have been used as a model to reproduce in vivo conditions. *H. parasuis* grown under these conditions have filamentous structures on the surface, which have been identified as fimbriae by electronic microscopy (Munch et al., 1992). However, the role of fimbriae for adhesion and as a virulence factor for *H. parasuis* needs to be clarified.

The presence of polysaccharide capsule is recognized as an important virulence attribute in preventing phagocytosis and bactericidal serum activity by complement. Encapsulated strains are potentially more virulent. However, the presence of capsule does not always correlate with virulence (Sandal et al., 2010). The presence of a

polysaccharide capsule on *H. parasuis* has been confirmed (Morozumi and Nicolet, 1986a), but it has not been purified or chemically characterized. Encapsulated and non-capsulated *H. parasuis* clones can be induced by in vivo or in vitro passages, respectively (Oliveira and Pijoan, 2002). Most of *H. parasuis* isolates from the upper respiratory tract of healthy pigs have been found to be encapsulated, whereas the majority of isolates from systemic sites of diseased pigs have been non-capsulated (Morozumi and Nicolet, 1986a). Different results were observed by Olvera et al. (2009), where *H. parasuis* strains isolated from polyserositis lesions had a prominent capsule and resisted phagocytosis, while capsule was not detected in strains isolated from the nose of healthy pigs. Therefore, the association of capsule with virulence is still controversial.

Lipopolysaccharide (LPS) is a prominent surface component of all Gram-negative bacteria. The term lipooligosaccharide (LOS) has become standard when referring to the LPS of bacteria from the genus *Haemophilus* because they lack polymerized O-antigen side chains (Sandal et al., 2010). Zucker et al. (1996) examined LOS production by *H. parasuis*, and found that virulent and avirulent strains shared similar patterns, which suggests that LOS might not be a good indicator of virulence.

Outer membrane proteins (OMPs) have been associated with virulence among *H. parasuis* strains. Potentially pathogenic *H. parasuis* isolates analyzed by polyacrylamide gel electrophoresis (PAGE) shared a 37 kDa major protein and were classified as PAGE type II, whereas isolates lacking this protein were classified as PAGE type I (Morozumi and Nicolet, 1986b; Ruiz et al., 2001). Pigs that survive *H. parasuis* challenge developed antibodies to OMPs but not to capsule or LOS, which

may suggest that antibodies against OMPs could be related to protection (Miniats et al., 1991). However, the role of *H. parasuis* OMPs as virulence factors or immunogens remains to be defined.

Hitherto, vaccination and antibiotics are the standard methods to prevent and control *H. parasuis* outbreaks. Seronegative pigs of all ages are extremely susceptible to Glasser's disease (Riising, 1981). Pigs acquire protective immunity against *H. parasuis* after recovery from systemic infection (Riising, 1981; Smart and Miniats, 1989; Miniats et al., 1991; Solano-Aguilar et al., 1999; Cerda-Cuellar et al., 2010). Moreover, there are numerous reports of successful control by vaccination with commercial or herd-specific (autogenous) bacterins (Smart and Miniats, 1989; Miniats et al., 1991; Kirkwood et al., 2001; Takahashi et al., 2001). There are also instances where bacterins are not efficacious. Lack of vaccine efficacy may be due to lack of cross-protection for the strain or serovar involved in the disease process or to inappropriate vaccination timing (Miniats et al., 1991; Rapp-Gabrielson et al., 1997).

Additionally, when vaccinating piglets against *H. parasuis,* it is important to consider interference of maternal antibodies with the development of active immunity by vaccinated pigs (Bak and Riising, 2002). Solano-Aguilar et al. (1999) demonstrated that vaccinated piglets born to vaccinated gilts were not affected by Glasser's disease after challenge with *H. parasuis*, while vaccinated pigs born to non-vaccinated gilts developed severe polyserositis. Additionally, Cerda-Cuellar et al. (2010) showed that maternal antibodies could help explain the absence of *H. parasuis* disease during the first weeks of life, even though most of the pigs are colonized shortly after birth. Vaccination of sows resulted in higher IgG levels in serum of pigs, with consequent

delay of *H. parasuis* colonization and reduction of heterogeneity of strains, compared with pigs born to non-vaccinated sows (Cerda-Cuellar et al., 2010).

According to Robinson (2004), colonization implies that the patient has a sufficiently high concentration of organisms at the site that they can be detected, without causing any clinical signs or symptoms. Colonization can persist for days to years, with resolution influenced by the immune response to the organism, competition at the site from other organisms and, sometimes, use of antimicrobials. The most important factor in determining if a patient is colonized or infected with an organism is the clinical presentation (Robinson et al., 2004). Smart et al. (1988, 1989) found that pigs from SPF herds could carry *H. parasuis* in the nose. However, Glasser's disease only affected SPF animals when they were mixed with apparently healthy conventional pigs carrying distinct *H. parasuis* strains. Introduction of *H. parasuis* into non-immune populations may result in high morbidity and mortality with spread of infection to swine of all ages without necessarily being associated with stress factors (Riising 1981). Vahle et al. (1995) reported that *H. parasuis* colonizes the nasal cavity as an initial event in the pathogenesis of *H. parasuis* infection of cesarean-derived colostrum-deprived (CDCD) swine. Nasal discharge and rhinitis after *H. parasuis* inoculation was followed by lesions of polyserositis and recover of the bacterium from nasal mucosa and systemic sites. Therefore, to determine the significance of *H. parasuis* isolates, one must consider the site of isolation and, most importantly, the clinical picture.

Controlled exposure has been adopted by some herds as an alternative method to control *H. parasuis* in the nursery. This method consist of identifying the prevalent *H.*

parasuis strains causing disease in a farm and exposing 3 to 5-day old piglets to a low dose of these live pathogenic strains. Field studies have demonstrated that this method may reduce nursery mortality by 50% (Oliveira et al., 2001a). The mechanism behind such protection was not determined but it was hypothesized that controlled exposure results in *H. parasuis* colonization and, in the presence of maternal immunity, may elicit a protective immune response without causing disease (Pijoan et al., 1997). This method was also effective at reducing nursery mortality compared to vaccination (Oliveira et al., 2004).

Appropriate use of antibiotics is considered an important component of Glasser's disease management either to treat disease or prevent it (Desrosiers et al., 1986). Pigs receiving antibiotic treatment early during infection with *H. parasui*s are usually able to survive systemic infection with this bacterium. Most *H. parasuis* strains are sensitive in vitro to antibiotics used in the swine industry (Aarestrup et al., 2004). The main antimicrobial agents used to treat swine respiratory infections include trimethoprim-sulfa, tetracycline, penicillin, ceftiofur, tulathromycin, ampicillin, lincomycin, tylosin, tiamulin and enrofloxacin. However, therapeutic options approved to treat *H. parasuis* are limited to enrofloxacin, ceftiofur, penicillin and tulathromycin (Papich and Riviere, 2009). Oral antibiotics have also been recommended as a preventive medication program for herds in which *H. parasuis* is a problem. Antibiotics may be effective at controlling *H. parasuis* infection by reducing morbidity and mortality, and improving clinical scores and growth parameters in treated pigs.

1.2 Protective Immunity

There has been a great expansion on knowledge in regards to the pig immune system and its effect in disease and vaccination. Immune response starts when microorganisms enter the host and engage the immune system. The innate immune system is the first line of defense, typically activated shortly after infection. Pattern-recognition receptors (PRRs), including the toll-like receptors (TLRs), monitor pathogen-associated molecular patterns (PAMPs) and induce different signaling pathways to activate the immune system against infection. The phagocytic cells of the innate immune system and production of various cytokines provide antimicrobial protection, recruit cells through the inflammatory process and assist in the activation of acquired immunity. The acquired immune response activation results in cytokine production, T-cell and B-cell activation, and antibody production. The acquired immune response also provides pathogen-specific memory for protection against subsequent infections with the same pathogen (Chase and Lunney, 2012).

Most information present in the literature describes the antibody-mediated immune response to *H. parasuis* (Riising, 1981; Miniats et al., 1991; Nielsen, 1993; Rapp-Gabrielson et al., 1997, Solano-Aguilar et al., 1999; Martin de la Fuente et al., 2009a), whereas little information is available for cell-mediated responses (Martin de la Fuente et al., 2009b; Frandoloso et al., 2012a).

1.2.1 Innate Defense Mechanisms

Innate immunity may be sufficient to protect a host against an invading agent or to prevent disease from occurring since it does not require previous exposure to antigen (Kindt et al., 2007). Innate immunity enables the pig to respond almost immediately to an infectious agent, controlling infection until activation of the adaptive immune

system (Roth, 2005). Intact skin and mucous membranes participate in the innate immune response by providing physical barriers to infection. The anatomical defenses are also associated with chemical barriers (lysozyme) and commensal microbiota that prevents colonization of body surfaces by pathogenic bacteria (Roth, 2005).

PRRs are a key component of the innate immune system. PRRs are receptors present in mammalian cells that are able to distinguish between PAMPs, which are conserved molecules present on microorganisms, such as LPS and peptidoglycans, and harmless antigens. TLRs are an example of these molecules (Uenishi and Shinkai, 2009). TLRs are present on cells belonging to the innate immune system, such as macrophages, neutrophils and dendritic cells. Innate immune responses are initiated by the contact between bacterial PAMPs and host TLRs followed by activation of NfκB signaling pathway. This activation leads to the activation of a transcription factor that turns on cytokine genes such as those for tumor necrosis factor-alpha (TNF-α), IL-1, and chemotactic attractants. The subsequent inflammatory response helps to activate other aspects of innate immunity and to initiate the acquired immune response (Akira and Hemmi, 2003).

Bacterial invasion will also be challenged by the activation of complement in blood and tissues and the stimulation of an inflammatory process that attracts both the innate and adaptive immune defenses to the site of invasion (Kindt et al., 2007; Ryan and Ray, 2010). The complement system participates in several innate immune reactions such as inflammation, phagocytosis and bacterial killing; however, it is also capable of causing serious damage to the host, if it is activated in an unregulated fashion. Complement is an enzymatic system composed of at least 20 serum proteins that are sequentially activated through one of two pathways, the classical and the alternative

pathways, involving the membrane attack complex and regulatory proteins. The classical pathway is activated by antigen-antibody complexes consisting of immunoglobulins IgG and IgM. The reaction between immunoglobulins and antigen activates the complement and initiates a cascade reaction on the surface of the microbe. The result of this reaction is the formation of pores in the cell wall that will lead to bacterial death. The alternative pathway of complement activation is mediated by certain bacterial products such as endotoxins (including bacterial LPS, peptidoglycan and teichoic acids). This allows antibody-independent activation of the complement cascade that is considered important in initial (pre-antibody) defense against various types of infections caused by bacteria (Kindt et al., 2007). Activation of any of the complement pathways cause vasodilatation and increased vascular permeability resulting in serum components (including antibodies and complement) entering the tissues to help control infection. Complement components produced during activation are chemotactic and attract phagocytic cells to the site of infection. They also coat or opsonize infectious agents to increase their uptake by phagocytic cells. Complement components also destroy pig cell membranes and some bacterial cell membranes (Chase and Lunney, 2012). Therefore, the complement system is important for mediating inflammation and controlling bacterial infections.

Little information is currently available for innate immune response to *H. parasuis.* Immunohistochemistry and in situ hybridization methods have demonstrated that following infection, *H. parasuis* is phagocytosed by neutrophils and macrophages and can be found as degenerated bacteria in dilated phagosomes (Amano et al., 1994; Segales et al., 1997). Moreover, a recent study showed that *H. parasuis* susceptibility to phagocytosis by porcine alveolar macrophages (PAMs) correlates with the clinical

origin of the strain (Olvera et al., 2009). *H. parasuis* strains isolated from systemic lesions (virulent) were resistant to phagocytosis, while nasal strains (non-virulent) were efficiently phagocytosed by PAMs in vitro, followed by subsequent bacterial death within the macrophage. Phagocytosis resistance by *H. parasuis* virulent strains is likely associated with presence of capsule (Olvera et al., 2009), which can interfere with complement deposition (Sandal et al., 2010).

Also playing an important regulatory role in modulating the immune responses, cytokines are proteins and glycoproteins that are secreted by cells and serve as intercellular signaling molecules. All cells of the immune system are capable of secreting and being influenced by cytokines. Cytokine secretion is usually transient, occurs in response to specific stimuli, and typically cytokines act locally in low concentrations. A cytokine will only act in a cell that has specific receptors for it. Regulation of cytokine receptor expression is an important mechanism for controlling the response to cytokines (Kindt et al., 2007). Most porcine cytokines that have been studied are similar to their orthologs in humans or mice (Chase and Lunney, 2012).

Some cytokines are important in mediating innate immunity. This includes the type I interferons (IFN-α/β) and the pro-inflammatory cytokines that include IL-1, IL-6, and TNF-α. Type I IFN production occurs in response to viral infection by many cell types. Type I IFN make cells resistant to viral infection, increase natural killer cell (NK) activity, and increase major histocompatibility complex (MHC) molecule expression on cell surfaces, thus increasing antigen presentation to T cells (Roth, 2005). The proinflammatory cytokines (IL-1, IL-6, and TNF-α) are produced primarily by macrophages in response to bacterial infection and require no previous exposure. They may also be produced in response to viral, protozoal, fungal

infections, or tissue damage. The proinflammatory cytokines stimulate the liver to produce acute-phase proteins. In addition, they induce fever, loss of appetite, and fatigue if present in high enough concentrations (Roth et al, 1997; Murtaugh and Foss, 2002; Chase and Lunney, 2012).

While the presence of cytokines is required for effective stimulation of immune responses, in large quantities they can induce hypovolemic shock and death (Kindt et al., 2007). Therefore, the adequate modulation of cytokine production is essential for control and elimination of bacterial infections. An infection model was created to study the expression of inflammatory cytokines during acute respiratory disease caused by *A. pleuropneumoniae* in swine. IL-1, IL-8, TNF and IL-6 mRNA were detected within 2 to 4 hours after bacterial infection by in situ hybridization and Northern blotting in all inoculated pigs (Baarsch et al., 1995; 2000). IL-1 might contribute to increased severity of disease, but elevated IL-6 levels were consistent with a protective acute phase response. The low TNF levels observed in this study were surprising, since TNF was hypothesized to be triggering the inflammatory cascade (Murtaugh et al., 1996). Another study demonstrated that macrophage activation involving high production of IL-1 and TNF was associated with severe lung injury in cases of *A. pleuropneumoniae* infection (Morrison et al., 2000). Increase of pro-inflammatory cytokines after infection with *A. pleuropneumoniae* indicated that cytokines were associated with the development of pleuropneumonia disease and contributed to disease injury (Morrison et al., 2000).

Similarly, *H. parasuis* was able to stimulate the production of proinflammatory cytokines IL-8 and IL-6 by pig tracheal cells and endothelial cells in an in vitro model

(Bouchet et al., 2008; 2009; Aragon et al., 2010). Acute phase response stimulated by IL-6 production and chemoattraction of leucocytes stimulated by IL-8 represent essential roles of these cytokines in inflammatory response to *H. parasuis*. Increased IL-1α expression in lung has been reported in pigs undergoing severe disease following experimental infection, whereas IL-4, IL-10, tumor necrosis factor-alpha (TNF-α), and (IFN-γ) were expressed in significantly higher levels in spleen, pharyngeal lymph nodes, lung and brain of survivors, which suggests that these cytokines might contribute to protection against *H. parasuis* (Martin de la Fuente et al., 2009c).

1.2.2 Acquired Immunity

While the porcine innate immune system confers initial protection, the acquired immune system provides a second, more specific and long lasting, line of defense against infectious organisms (Lunney, 2005). In non-immune animals, precursor T cells exist as "resting T cells", bearing T-cell receptors (TCRs) for specific antigens. Various innate signals, such as TLR signaling and cytokines, attract immune cells to the local tissues where they undergo activation. These immune cells then use their surface molecules to signal each other's activation (Lunney, 2005). Foreign antigens must be processed by antigen presenting cells (APCs) to stimulate adaptive immunity. The induction of clonal expansion of the immune response requires complex interactions of macrophages, T, and B-lymphocytes (Kindt et al., 2007).

Following antigen stimulation, CD4+ T cells differentiate into T helper (Th) cells with a characteristic cytokine secretion profile. The mice Th-1 patterns of cytokine production include IL-2 and IFN-γ, whereas Th-2 cells produce IL-4, IL-5, IL-6, IL-9,

IL-10 and IL-13. Some cytokines are secreted by both Th-1 and Th-2, including IL-3, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Mosmann and Sad, 1996). The functions of Th-1 and Th-2 cells correlate with the production of their cytokines. Th-1 cytokines are involved in cell-mediated inflammatory functions, while Th-2 cytokines encourage antibody production, and also enhance eosinophil proliferation and function (Mosmann and Sad, 1996).

The number of studies conducted on porcine cellular immune responses to infectious microorganisms has increased in recent years, but it is still limited, in comparison with work performed on other species. Specific reagents, improved technology and a more detailed knowledge of the porcine immune cell populations now enables better association of the interactions between pathogens and the porcine immune system, and detailed analyses of the antigen-specific T-cell response (Saalmuller, 1998). Although protective immunity against extracellular bacteria is mainly dependent on antibodies, T cell responses are often required for full expression of immunity (Chase and Lunney, 2012).

In order to stimulate the adaptive immune response, the foreign antigen must be processed by an APC. APCs internalize bacterial agents and then present antigenic fragments bound to MHC II molecules on the surface of the cell. This antigen-MHC II complex is presented to a Th-2 cell, which can only efficiently recognize foreign antigens that are on the cell surface bound to MHC class II molecules. In addition to antigen-MHC II complex, the Th cell requires the presence of cytokines released by APCs and other T cells, and contact with co-stimulatory molecules on the surface of the APC for complete activation. Following antigen presentation, the macrophage will

secrete IL-1, which will activate Th-2 cells to secrete IL-2. IL-2 will induce Th-2 cells to proliferate. This process continues with stimulated Th-2 cells secreting a variety of other cytokines including IL-2, IL-4, IL-6, and IFN-γ. IL-4 causes B cells to proliferate and differentiate into antibody-secreting plasma cells and memory B cells (Lunney, 2005; Kindt et al., 2007; Chase, 2012). In pigs, however, IL-4 seems to play a different role than in mice. Porcine IL-4 was not able to stimulate porcine B cell and it blocked antibody production. Porcine IL-4 did have a stimulatory effect on lymphoblast's cell growth (Murtaugh et al., 2009). Therefore, the role of IL-4 in porcine immunology needs further investigation. Appleyard et al. (2002) evaluated possible correlates of protection in blood lymphocyte subset phenotypes of pigs receiving either a commercial *A. pleuropneumoniae* bacterin, low dose (LD) of aerosol infection with the same bacteria, or control group. All pigs were subsequently challenged with a high dose of *A. pleuropneumoniae*. B-cells increased following vaccination. CD4+ lymphocytes increased significantly in both vaccinated and LD groups after challenge, while CD8+ cells decreased in the LD-group (Appleyard et al., 2002).

Specific changes in peripheral blood mononuclear cells (PBMC) were found in colostrum-deprived pigs after challenge with virulent *H. parasuis* strain, but not after immunizations (Martin de la Fuente et al., 2009b; Frandoloso et al., 2012a). These changes consisted mainly of increase in the relative proportions of T and B-lymphocytes in immunized pigs that survived challenge (Martin de la Fuente et al., 2009; Frandoloso et al., 2012a). As extracellular pathogens, *A. pleuropneumoniae* and *H. parasuis* would be expected to require a dominantly Th-2 response to mediate host resistance. Several studies show that bactericidal antibody production appears to

correlate with protection (Ryan and Ray, 2010). Yet, the appearance of the different subsets of T lymphocytes during infection with these two bacteria might also be relevant to protection.

Helper lymphocytes are also critical in initiating B-cell responses, which result in antibody production. B cells contact antigen through antibodies bound to their surface, which act as B-cell receptors (BCRs). Contrary to T-cells, B-cells can react with soluble antigens, without having to be presented on MHC class II molecules by APCs. However, optimal B-cell response to antigen requires Th-cell release of cytokines. When a B-cell is bound to antigen, and simultaneously is stimulated by IL-4 produced by a nearby Th-2 cell, the B cell undergoes mitosis and clonal expansion. At the same time, B cells differentiate into plasma cells or memory cells and experience class switching of antibody production from IgM to IgG, IgA, or IgE. The antibodies secreted by plasma cells react specifically with the homologous antigen that induced their formation. Even though they produce large amounts of antibodies, plasma cells are relatively short-lived (about one week). Therefore, B-cells also differentiate into memory cells, which are relatively long-lived and upon subsequent exposure to antigen, they become quickly transformed into antibody-producing plasma cells (Kindt et al., 2007).

Development of antibodies against *H. parasuis* has been demonstrated in convalescent and vaccinated pigs by complement fixation, western blot analysis, and ELISA testing (Miniats et al., 1991; Nielsen, 1993; Solano-Aguilar et al., 1999). Pigs exposed to *H. parasuis* live cultures or vaccinated with killed bacterins generate a transient immunoglobulin M (IgM) response followed by a solid and progressively

increasing IgG antibody response. Pigs with high titers are protected against challenge (Martin de la Fuente et al., 2009a), while Glasser's disease has been associated with absence or low titers of serum antibodies (Riising, 1981; Rapp-Gabrielson et al., 1997). Moreover, passive immunization of pigs with serum containing specific antibodies against *H. parasuis* was demonstrated to have protective effects against lethal challenge (Nedbalcova et al., 2011). In vitro studies suggested that antibodies play an important role in opsonization of virulent *H. parasuis* strains to facilitate phagocytosis (Olvera al., 2009). Virulent *H. parasuis* strains required prior opsonization with specific antibodies in order to be phagocytosed by PAMs and, if internalized, they were killed by PAMs (Olvera et al., 2009).

Maternal antibodies are a critical factor protecting pigs against the development of systemic infection and, apparently, there is no interference with the pig's active immunity (Solano-Aguilar et al., 1999). Additionally, pigs lacking maternal immunity were susceptible to Glasser's disease upon inoculation with a virulent *H. parasuis* strain, whereas pigs that received maternal immunity were protected (Blanco et al., 2004). Cerda-Cuellar et al. (2010) further demonstrated the importance of a good balance between colonization and immunity to avoid systemic disease caused by *H. parasuis*. Piglets from vaccinated sows had significantly higher levels of antibodies earlier after birth, and were colonized later and to a lower degree than piglets from non-vaccinated sows. The peak of *H. parasuis* nasal colonization for both groups was at 60 days of age, when *H. parasuis* was isolated from the nose of 100% of piglets from non-vaccinated sows and 85.7% of pigs from vaccinated sows. Furthermore, the increase in colonization rate was associated with a decrease in *H. parasuis* serum

antibodies in piglets, which indicates that the level of maternal antibodies in piglets might be able to modulate the timing and level of colonization by *H. parasuis*.

Besides causing systemic disease, *H. parasuis* is also commonly isolated from the upper respiratory tract of healthy pigs. Consequently, *H. parasuis* might interact with the mucosal immune system, possibly resulting in priming of the immune response. Indeed, pigs exposed to aerosol containing live non-pathogenic *H. parasuis* cells developed serum antibodies and resisted challenge with virulent *H. parasuis* strains (Nielsen, 1993). Moreover, oral exposure of 5-day-old pigs to a low dose of a live *H. parasuis* strain significantly reduced mortality compared to vaccination (Oliveira et al., 2004).

In addition to serum antibodies, secretory IgA (sIgA) serves as an important protective mucosal element. The B cells that produce IgA preferentially migrate to submucosal tissues where they differentiate into plasma cells that secrete IgA. SIgA antibodies bind to the poly-Ig receptor on the basolateral surface of respiratory epithelial cells, and are transported to the mucosal surface of the epithelial cell. The cleavage product is called the secretory component and remains bound to the dimeric IgA (Snoeck, et al., 2006). Contrary to serum IgA antibodies, sIgA is not able to trigger phagocytosis, which is presumably due to blockage of Fc part of the IgA heavy chain by the secretory component. However, sIgA antibodies contribute to the protection of the mucosal epithelial barrier through other mechanisms. SIgA antibodies help clear pathogens by preventing adherence of microorganisms to epithelial cells. Dimeric sIgA is still more efficient than IgA because it can cross-link large antigens with multiple epitopes. Complexes of sIgA and antigen are easily

entrapped in mucus and then eliminated by the ciliated epithelial cells (Snoeck, et al., 2006).

Little is known about the mucosal immune response to *H. parasuis* in the respiratory tract of pigs. Levels of IgA increased in nasal secretions and bronchoalveolar lavage (BAL) fluid from pigs vaccinated intranasally with a subunit vaccine containing recombinant virulence associated trimeric autotransporter (VtaA) and it was associated with partial protection against *H. parasuis* challenge (Olvera et al., 2011). In a different study, specific IgA against *H. parasuis* was detected in serum of pigs immunized with a subunit vaccine when the vaccine was delivered intratracheally, but not when it was delivered intramuscularly. However, the presence of IgA in the respiratory tract was not evaluated (Martinez-Martinez 2012).

1.3 **Effects of antimicrobials on the immune response**

Antimicrobials have been widely used in the swine industry since they were discovered over 50 years ago. The use of antimicrobial agents during all phases of pig's growth is one of the most cost-effective tools in the efficient production of pork. Increase in disease challenge due to the decline of small farms and increase in the larger ones, particularly in the USA, is associated with an increase of the use of antimicrobials in pork production (Friendship, 2000). The emergence and re-emergence of viral diseases, particularly porcine reproductive and respiratory syndrome (PRRS) virus and porcine influenza virus which are often found interacting with bacterial agents has also been associated with an increased use of antimicrobials to control swine respiratory disease (VanAlstine, 2012).

Antimicrobials are mainly used in the swine industry for growth promotion, disease prevention and treatment (Cromwell, 2002). Antimicrobials in swine have also been used as part of disease elimination programs for pathogens such as *Mycoplasma hyopneumoniae* (Dee, 1994, Rautiainen et al., 2001) and *A. pleuropneumoniae* (Andersen and Gram, 2004). However, pathogen elimination has not always been successful, in particular for pathogens such as *H. parasuis* and *Streptococcus suis,* considered part of the commensal flora of the pig (Clark, 1994). The majority of antimicrobial drugs used in swine farms are incorporated into feed for growth promotion (subtherapeutic use) (Friendship, 2000).

The present review will deal primarily with antimicrobial drugs as therapeutic agents. The in vivo response of a host and bacteria to an antimicrobial agent is a product of the interaction of many factors including susceptibility of the bacteria to the agent, age and immune status of the host, existing disorder, and route of antimicrobial administration (Ngwai et al., 2011). Therefore, selection of the adequate antimicrobial agent depends on the antimicrobial sensitivity pattern of the pathogenic organism, the established MICs of the antimicrobials being considered, the legislation concerning its use, and the drug's cost (Friendship, 2000).

According to data from the National Animal Health Monitoring System (NAHMS) Swine 2006 study collected from 514 swine production sites, the most common antimicrobials given by injection to nursery-age pigs for disease treatment or prevention were ceftiofur and procaine penicillin G (43 and 43.9 percent of sites, respectively). About half of sites with nursery-age pigs (48.6 percent) used injectable

antimicrobial to treat respiratory disease and the most common actions taken for pigs with clinical respiratory disease were to administer antimicrobials to all pigs in the entire room with clinically ill pigs (39.6 percent of sites). Therefore, the use of antibiotics in swine is widespread, not only as growth promoters in the feed, but also to control and treat disease by intramuscular administration. While it is clear that the use of antimicrobials is an important tool to control disease in pigs, its potential effect on the immune response is less understood.

1.3.1 Effect of antimicrobials on bacterial infection and immune response

The widely known activity of antimicrobial agents is the direct (bacteriostatic or bactericidal) effect against microorganisms. Antimicrobials decrease the bacterial load and thereby permit the host to activate immune defenses and eliminate the pathogen without excessive inflammation (Walker, 2000; Labro, 2000). At the same time, early elimination of the pathogen by antibiotics may hinder the development of a protective immune response necessary to overcome future infections (Su et al., 1999; Griffin et al., 2009). Additionally, antimicrobial use in swine may affect the commensal microbiota with unpredictable effects on disease dynamics (Macedo et al., 2012; Vilalta et al., 2012).

In regards to the effect that antimicrobials may have on the development of an immune response, early elimination of bacterial infection has been reported to have an effect on the immune response activation (North et al., 1981; Su et al., 1999; Griffin et al, 2009; Sjolund et al., 2009; Johanns et al, 2011). An undesirable effect of antimicrobial-induced elimination is the substantial reduction in the generation of immunologic cell-mediated memory. In the case of *Listeria monocytogenes* infection

in mice, when ampicillin was employed to rapidly eliminate infection at any time during the first 5 days post infection, there was a great reduction in the capacity of antibiotic-treated mice to resist a subsequent challenge. The earlier the primary infection was eliminated, the lower the level of immunologic memory generated. Additionally, when ampicillin was used to eliminate remaining bacteria at the time of peak response (day 6 after infection), the rate of decay of immunity, as measured by the rate of loss of protective T cells from the spleen, was greatly increased. Therefore, the magnitude and duration of the T cell-mediated anti-*Listeria* immune response were determined by the level and duration of infection with live, replicating antigen (North et al., 1981).

Similarly, other studies have shown that early antibiotic treatment can prevent the development of protective immunity against reinfection with *Chlamydia trachomatis* and *Salmonella* sp. (Su et al., 1999; Griffin et al, 2009). Mice that can spontaneously resolve primary infection with *C. trachomatis* exhibit significant resistance to reinfection and that correlates with the production of local IgA and IgG, and with a chlamydial-specific CD4+ Th1 cell–mediated immune response (CMI) (Williams et al., 1997). In contrast, treatment with doxycycline at the time of infection (day 0) and at days 3, 7, and 10 post infection, resulted in a significant reduction in chlamydial-specific antibody, CMI responses and protection against secondary chlamydial genital infection (Su et al., 1999).

Antimicrobials used to eliminate primary *Salmonella sp.* infection also rendered mice susceptible to secondary infection against *Salmonella sp*. Protection from *Salmonella sp.* requires *Salmonella*-specific Th1 cells. The optimal development of that protective

immunity requires at least two weeks of exposure to the live attenuated *Salmonella sp.* strain (Griffin and MacSorley, 2011).Antibiotic treatment in the water with enrofloxacin for 5 weeks beginning 2 days post infection not only prevented the development of an effective immune response but also rendered the animals susceptible to challenge infection. Even though the specific immune response was activated rapidly in antibiotic-treated mice, it was not sustained after successful antibiotic treatment (Griffin et al., 2009). In contrast, a different murine model of persistent *Salmonella* infection demonstrated that water treatment with enrofloxacin starting as early as 5 days post infection eliminated primary infection without rendering mice susceptible to the secondary infection. Interestingly, in this case protection was not mediated by CD4+ or CD8+ T cells because depletion of these cells either alone or in combination prior to the second challenge did not abrogate protection. Instead, robust levels of *Salmonella*-specific antibody were primed and were shown to be protected when transferred to naïve mice (Johanns et al, 2011).

One study has been performed on the effect of antibiotic treatment on the immune response in swine. In the case of *A. pleuropneumoniae*, Sjolund et al. (2009) investigated the susceptibility to an initial challenge and re-challenge with *Actinobacillus pleuropneumoniae* in SPF pigs that were treated with antimicrobials (penicillin, enrofloxacin or tetracycline) at the onset of clinical signs. After initial exposure to *A. pleuropneumoniae,* the inoculated control and the penicillin-treated groups developed severe disease, but the groups treated with enrofloxacin and tetracycline recovered rapidly. All the inoculated pigs, except those treated with enrofloxacin, developed serum antibodies to *A. pleuropneumoniae*, which protected them against the second challenge (Sjolund et al., 2009). Therefore, in this case the

development of a protective immune response was impeded by treatment with enrofloxacin but not by treatment with penicillin or tetracycline.

There is no specific information available on the role of antibiotics on the immune response to *H. parasuis.* However, fluoroquinolones have been associated with potential interference with the dynamics of respiratory colonization of bacterial pathogens including *H. parasuis,* in pigs (Le Carrou et al., 2006; Macedo et al., 2012; Vilalta et al., 2012). Fluoroquinolones are used to treat respiratory diseases (Papich and Riviere, 2009). These drugs are known for their high efficiency and very low levels of resistance. In the case of *H. parasuis*, intramuscular administration of marbofloxacin was able to reduce the nasal carriage of *H. parasuis* in weaned pigs (Vilalta et al., 2012). In another study, enrofloxacin reduced the numbers of *H. parasuis* in the tonsil and nasal cavity of pigs and decreased the number of positive pigs during the first week after treatment (Macedo et al., 2012). Both, marbofloxacin and enrofloxacin, showed a similar effectiveness after treatment, being able to reduce the amount of *H. parasuis* in the upper respiratory tract of pigs but not to eliminate it. As mentioned in a previous section of this manuscript, reduction in *H. parasuis* colonization at a young age has been associated with the development of Glasser's disease during the post-weaning period (Pijoan et al., 1997, 2003; Oliveira et al., 2001a, 2004). Therefore, antibiotic treatments that interfere with *H. parasuis* colonization in young pigs could result in increased disease susceptibility at an older age. However, no studies have been performed on this topic.

1.3.2 Antimicrobial Immunomodulation

In addition, some antimicrobials can directly modulate innate and adaptive immune responses. Antimicrobial agents such as beta-lactams, quinolones, and macrolides, are commonly used in the treatment of respiratory tract infections in swine. In addition to their potent direct antibacterial activity, some of these antimicrobials, especially macrolides and fluoroquinolones, have immunomodulatory effects. Immunomodulation refers to the action undertaken by medication on processes that guide the immunological defense system. The immunomodulatory capabilities of antimicrobial agents have been demonstrated in human cells and, to a lesser extent, in animal experiments, especially mice (Zimmermann et al., 2009).

For the purpose of this review, the immunomodulatory effects of quinolones will be addressed. Quinolones have been demonstrated to beneficially interact with the immune system in vitro and in animal models (Riesbeck, 2002). Specifically, the effects of quinolones on porcine phagocytic activity and cytokine production are presented below.

Fluoroquinolones are actively accumulated in phagocytes. At clinically achievable concentrations, quinolones might affect granulocyte functions (e.g., phagocytosis or chemotaxis). Bacteria pre-incubated with quinolones are also more easily phagocytized compared to untreated controls (Riesbeck, 2002). Enrofloxacin is a fluoroquinolone routinely used for the treatment of respiratory bacterial infections in pigs and other livestock species and poultry. In a study evaluating the effects of enrofloxacin on porcine phagocytic function, this antimicrobial was shown to accumulate in porcine polymorphonuclear leukocytes (PMNs) and alveolar

macrophages (AMs) when clinically relevant concentrations of enrofloxacin were used (Matera et al., 1996; Garcia et al., 1992). In addition, intraphagocytic killing of *A. pleuropneumoniae* was significantly enhanced by enrofloxacin in both PMNs and AMs (Schoevers et al., 1999). More research is needed to investigate whether these effects would also apply to other swine bacteria such as *H. parasuis*.

Fluoroquinolones have also been shown to have modulatory effects on cytokine release. Cytokines are essential elements of the effective immune response against infections in general. However, prolonged production of cytokines may result in exacerbation of inflammatory reactions and tissue damage (Williams, 2005). As explained before, cytokine production represents one immunological factor that directs the particular type of immune response by controlling the differentiation of precursor T helper (Th0) cells into Th1 or Th2 cells. As a result, antimicrobials that affect cytokine production may alter the Th response induced by microbial infection. Williams et al. (2005) have shown that the quinolone agents, moxifloxacin and ciprofloxacin, have pronounced effects on Th1 and Th2 cytokine expression in human mononuclear cells. Both quinolones decreased the number of cells expressing IL-4 and IFN-γ in stimulated cells compared to control cells without antimicrobials. Despite the changes in the number of cells expressing IFN- γ and IL-4, there was no significant change in the ratio of the number of INF-γ-positive to IL-4-positive cells (Th1/Th2 ratio) in cells exposed to moxifloxacin or ciprofloxacin. These results have shown that both quinolones can have effects on Th cell cytokines, with potential implications for immune response and recovery after severe infection, but the mechanisms of such effects need further investigation.

Additional control of cytokine production by fluoroquinolones may have a significant role during septic shock. Septic shock that results from Gram-negative bacterial infections including *H. parasuis* has been reported in pigs (Post, 2012). Two important mediators of septic shock are tumor necrosis factor (TNF) and interleukin-1 (IL-1); inducing changes that are similar to those induced by endotoxin (Khan et al., 2000). Although concentrations of cytokines fluctuate during sepsis, TNF, IL-1, IL-6, and IL-10 are frequently elevated in individuals in septic shock (Khan et al., 2000). In vitro studies have shown that the fluoroquinolone trovafloxacin reduces cytokine production by lipopolysaccharide (LPS)-treated human monocytes, especially IL-1, IL-6, IL-10 and TNF-α (Khan et al., 1998). Moreover, the fluoroquinolones ciprofloxacin, trovafloxacin and tosufloxacin have been shown to significantly reduce the IL-6 and TNF-α serum concentration in quinolone-treated mice injected with LPS, and increase the survival of mice receiving lethal dosages of LPS (Khan et al., 2000). The mechanisms by which these antimicrobials contribute to protection in LPS-injected mice are not clear (Khan et al., 2000).

1.4 Summary

H. parasuis is considered one of the most important bacterial pathogens affecting pigs. Vaccines and other management strategies have not always been successful in controlling the losses associated to *H. parasuis.* The success of *H. parasuis* controlled exposure in preventing mortality during the nursery stage suggests that early colonization with virulent *H. parasuis* protects pigs from developing disease. We speculate that colonization primes a protective immune response. Therefore, factors that may interfere with colonization may interfere with the development of an effective immune response. Furthermore, the use of antibiotics has been shown to

alter *H. parasuis* colonization patterns. We hypothesize that disruption of *H. parasuis* colonization by antibiotics might hinder the development of priming immunity by rapidly eliminating the source of bacterial antigens.

We reviewed information to determine whether disruption of colonization could be important in Glasser's disease. Recent publications show that antibiotics can prevent the development of protective immune response against infection with agents, such as *A. pleuropneumoniae, C. trachomatis* and *Salmonella* sp. Because antibiotic use is widespread in the swine industry and it is used as an option to control *H. parasuis* disease, we speculate that selected antibiotic schemes may result in subsequent *H. parasuis* associated disease. Moreover, serum antibody response is important for protection against *H. parasuis,* even though little is known about immune response to *H. parasuis* colonization. Furthermore, although there are studies showing that antibiotics reduce nasal colonization with *H. parasuis,* there is no information on the effect of antibiotics on *H. parasuis* immunity. An improved understanding of whether *H. parasuis* colonization may prime an immune response that will be protective and whether this response may be impaired by the use of antibiotics will contribute to the development of better control programs for *H. parasuis* in the field and will help develop judicious antibiotic treatment practices.

CHAPTER 2

ROLE OF OLIGOPEPTIDE PERMEASE A (OPPA) PROTEIN IN HUMORAL IMMUNE RESPONSE TO *HAEMOPHILUS PARASUIS* IN PIGS

The work has been submitted to:
Macedo N, Oliveira S, Torremorell M, Rovira A. Role of Oligopeptide permease A (OppA) protein in humoral immune response to *Haemophilus parasuis* in pigs. Veterinary Microbiology.

Abstract

Haemophilus parasuis is an important swine pathogen that causes Glasser's disease, characterized by pneumonia, polyserositis and meningitis. Protection against *H. parasuis* infection is associated with the presence of homologous antibodies in serum. However, a *H. parasuis* antigen that can elicit a protective immune response against all *H. parasuis* strains has yet to be found. A novel immunogenic and species-specific *H. parasuis* protein was identified by screening *H. parasuis* whole cell proteins using swine convalescent sera. One protein of 52 KDa was clearly immunodominant and conserved among different *H. parasuis* strains. This protein was further identified as an oligopeptide permease A (OppA). Because OppA elicited a specific antibody response in pigs that recovered from *H. parasuis* infection, we investigated its potential role in diagnostics and protective immunity. An ELISA test using recombinant OppA as its coating antigen was further developed and tested. *H. parasuis* specific antibodies to rOppA were detected in serum from convalescent pigs but not in serum from specific pathogen free (SPF) or conventional pigs. Pigs immunized with rOppA protein had –antibody in serum against rOppA. However, challenged pigs were not protected against challenge with pathogenic H. parasuis. We conclude that OppA is a universal species-specific *H. parasuis* immunogen, and a good marker for previous systemic infection with *H. parasuis.*

Introduction

Haemophilus parasuis is an important swine pathogen that causes Glasser's disease, a systemic disease characterized by pneumonia, polyserositis and meningitis (Oliveira and Pijoan, 2004). High mortality and morbidity caused by *H. parasuis* infections are common, often in association with viral infections, such as porcine reproductive and respiratory syndrome virus (PRRSV) and influenza A virus (IAV) (Solano et al., 1998; Oliveira and Pijoan, 2004). Control of Glasser's disease is complicated by the lack of cross protection between the multiple genetically distinct strains, and the limited knowledge on virulence factors and protective antigens (Costa-Hurtado and Aragon, 2013). The lack of a consistent and accurate serological test to characterize the development of protective antibodies in sows and piglets also makes the control of *H. parasuis* a permanent challenge.

Protection against *H. parasuis* infection is associated with the presence of homologous antibodies against this pathogen (Martin de la Fuente et al., 2009a; Nedbalcova et al., 2011). Current commercial vaccines are usually based on bacterin extracts of a few strains that do not provide complete cross-protection against all the different strains (Bak and Rising, 2002; Hoffman and Bilkei, 2002). Recently, subunit vaccines based on immunogenic proteins have gained interest. Specifically, antibodies to outer membrane proteins, but not to LPS or capsule, have been associated with protection (Miniats et al., 1991). Vaccination of mice or pigs with vaccines containing newly identified immunogens such as PalA, Omp2, D15 and HPS-06257 (Zhou et al., 2009), recombinant transferrin-binding protein (rTbp) (Frandoloso et al., 2011), VtaA (Olvera et al., 2011), rSmpA, rYgiW and rFOG (Yuan et al., 2011), rGAPDH, rOapA and rHPS0675 (Fu et al., 2013), resulted in partial protection only. Therefore, the

identification of novel *H. parasuis* immunogens is still needed for the development of efficacious control methods for *H. parasuis.*

Oligopeptide-biding proteins belong to the ATP-biding cassette (ABC) family of transporters (Monnet, 2003). Oligopeptide permease A (OppA) protein, one of several subunits of ABC transporters, is a transmembrane protein in Gram-negative bacteria. Previous studies have reported the ability of OppA to elicit immune responses against a variety of bacteria, including *Yersinia pestis* (Tanabe et al., 2006) and *Borrelia burgdorferi* (Nowalk et al., 2006). Specifically for *H. parasuis,* OppA has been shown to induce an immune response in immunized mice (Hong et al., 2011). However, the immune response against *H. parasuis* OppA in the natural host, the pig, has not been investigated. Furthermore, the ability of such an immune response to protect pigs from *H. parasuis* disease remains unknown. In this study, we identified the immune response against *H. parasuis* OppA in pigs after they survived a systemic infection under field conditions. We also evaluated the potential of OppA as an ELISA antigen candidate and its capacity to confer protection to pigs against *H. parasuis* lethal challenge.

Material and Methods

Farm and pig selection, sample collection and H. parasuis isolation

A North American swine herd experiencing high nursery mortality (>5%) was selected. Clinical signs in affected nursery pigs included fever, respiratory distress, lameness, and central nervous system signs. Lesions included mostly fibrinous polyserositis. Glasser's disease was confirmed by culture of *H. parasuis* from affected organs. Mortality was mainly observed between 5-6 weeks of age.

During a first farm visit, 20 pigs presenting clinical signs of *H. parasuis* disease were selected. Pig selection was based on clinical signs characteristic of *H. parasuis* infection (described above) and rectal temperature higher than 105° F. Ten pigs were euthanized and necropsied, and the other 10 pigs were bled and treated with ceftiofur crystalline free acid (Excede® for swine, Zoetis, Florham Park, New Jersey, USA), at 5 mg/kg of body weight, intramuscularly. Samples for *H. parasuis* isolation included swabs from heart, brain, pleura, lung, joint and pericardium. In a follow up visit three weeks later to the same farm, convalescent sera were collected from the pigs treated with ceftiofur.

After collection, swabs were placed in Stuart medium and transported under refrigeration to the laboratory, where they were cultured onto 5% sheep blood agar plates with a nurse *Staphylococcus aureus* streak. All plates were incubated at 37°C in a 5% CO_2 atmosphere. Plates were checked for the presence of *H. parasuis* suspect colonies at 24 and 48 hours. Suspect colonies showing satellitism to the *S. aureus* nurse streak were isolated onto a new blood agar plate, incubated in similar conditions for 24 additional hours, and characterized through biochemical testing. DNA from pure cultures was extracted using PrepMan™ Ultra Sample Reagent following the manufacturer's instructions and tested by Polymerase Chain Reaction (PCR) as described by Oliveira et al. (2001b) to confirm the identity of the pathogen isolated. Cultures were harvested and kept frozen at -80°C until use.

Genotyping and serotyping

To evaluate the genetic and phenotypic *H. parasuis* variability in this herd, and to identify the prevalent *H. parasuis* strain, *H. parasuis* isolates were genotyped using Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR (Oliveira et al., 2003b) and serotyped by immunodiffusion test using heat stable cell extracts (Raffie and Blackall, 2000). Upon completion of ERIC-PCR genotyping, a representative isolate from each strain group was selected to be included in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis for protein identification.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Whole cell protein profiles of *H. parasuis* field isolates representative of each strain group and *H. parasuis* reference strains were evaluated using SDS-PAGE (Kawai et al., 2004). All *H. parasuis* isolates were grown under the same conditions described above. Overnight cultures were suspended in 1 ml of PBS and vortexed vigorously for 1 minute. Each suspension was diluted in PBS to achieve a final concentration of 1 µg/µl, measured by spectrophotometer. Fifty microliter of this suspension was added to 50µl of sample buffer containing 47.5µl of Laemmli Sample Buffer (Bio Rad Laboratories, Hercules, CA) and 2.5µl of 2-Mercaptoethanol (Bio Rad Laboratories, Hercules, CA). The final solution was boiled for 3 minutes for complete dissociation. For SDS-PAGE, 40 µl of the boiled solution was loaded per lane of a gel containing 4% stacking gel and 10% separating gel (Protean Ready Precast Gel, Jule, Inc, Milford, CT). Electrophoresis was carried out in a Protean® II xi Cell equipment (Bio Rad Laboratories, Hercules, CA) with a current of 25mA for 30 minutes and 35mA for 4 hours. The proteins were visualized by staining with Coomassie Brilliant Blue

R-250 1x solution and then destained with Comassie R-250 Destaining solution 1x (Bio Rad Laboratories, Hercules, CA).

Western blot analysis using convalescent sera

Identification of whole cell proteins recognized by the immune system of pigs that survived the *H. parasuis* outbreak was evaluated using Western blot analysis according to Towbin et al. (1979). Briefly, following SDS-PAGE analysis, proteins of *H. parasuis* field isolates and reference strains were transferred to PVDF membranes. PVDF membranes were blocked with 5% non-fat dried milk (NFDM) (Bio Rad Laboratories, Hercules, CA) in Tris-buffered saline (TBS) (Bio Rad Laboratories, Hercules, CA) for 2 hours. Sera collected from convalescent pigs were used as primary antibodies at 1:50 dilution and incubated with the membrane for 1 hour. The membrane was subsequently incubated for 1 hour with anti-Pig IgG-Peroxidase antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:1000 as secondary antibody. The membranes were stained using the Immun-Blot® Opti-4CN™ Colorimetric Kit (Bio Rad Laboratories, Hercules, CA Bio-Rad) following the manufacturer's instructions. After staining the membranes, pictures were taken with a digital camera and the membranes were let dry completely. All membranes were stored refrigerated. Serum from 1-day-old piglets collected prior to suckling was used as negative control for non-specific binding. Western Blot using whole cell proteins extracted from bacterial pathogens commonly isolated from swine was performed for specificity. Specificity testing included *Pasteurela multocida, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Actinobacillus idolicus, Actinobacillus minus, Actinobacillus suis, Actinobacillus porcinus, Escherichia coli, Salmonella choleraesuis,* and *Streptococcus suis.*

Protein identification, cloning and expression

An immunodominant protein recognized by convalescent antibodies from naturally infected pigs was identified and sequenced. N-amino-terminal sequencing was performed directly from this membrane-bound protein utilizing Edman degradative chemistry (Oligonucleotide and Peptide Synthesis Facility, BioMedical Genomics Center, University of Minnesota). Analysis of protein sequences within GenBank and BLAST (Altschul et al., 1990) searches were conducted using NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

After protein sequencing and identification, the gene encoding for the immunogenic protein specific for *H. parasuis* was purified, cloned and expressed in *E. coli.* (GenScript Corp., Piscataway, New Jersey). Briefly, a synthetic codon-optimized version of the immunogenic protein gene was prepared using the OptimumGene™ - Codon Optimization technology. A pET-derived vector that attaches an N-terminal polyhistidine tag to the protein was used and optimized for expression in *Escherichia coli*. Expression was then induced using IPTG and soluble proteins were isolated by Ni–NTA metal-affinity chromatography (Burgess-Brown et al., 2008). The N-terminal region and tag were then removed by cleavage to obtain high-purified protein. The recombinant protein was then analyzed by SDS-PAGE, Western blot and sequencing to confirm its identity.

ELISA test development

After expression of the identified protein specific for *H. parasuis,* this recombinant antigen was used to develop an indirect ELISA test to detect antibodies against this

protein. A checkerboard titration was performed to determine the optimal working dilution of the coating antigen, serum and anti-pig IgG-Peroxidase conjugate (Sigma-Aldrich) (HRP-IgG) (Sigma) using a 96-well ELISA plate (Costar 3590 High biding 96 well EIA/ RIA Plate, flat bottom, Corning Incorporated). The dilutions that gave the maximum difference between positive and negative sera by absorbance at 450 nm were selected for testing of serum samples.

After optimization, indirect ELISA was performed using the following procedure. One hundred nanograms of antigen were diluted in carbonate buffer and each of the plate wells were coated with 100 µl. Plates were washed with solution of phosphate buffer saline and tween 20 (TPBS) pH 7.4 at room temperature using a microplate washer (ELx405^{TM} Biotek) after each incubation step. The plates were then incubated with 300 µl/well of 5% non-fat dried milk (NFDM) in TPBS and allowed to stand for 2 hours at room temperature. Serum samples were diluted 1:50 in 5% NFDM in PBST and 100 µl was added to each well and then incubated for 1 hour. Anti-pig IgG-peroxidase conjugate (Sigma-Aldrich) was diluted 1:100000 in 5% NFDM in PBST and 100 µl were added to each well and incubated for 1 h. Equal volumes of TMB peroxidase substrate and peroxidase H_2O_2 (KPL, Gaithersburg, MD) were mixed together and 100 µl were added to each well. The reaction was quenched by adding 100 µl of 1 M phosphoric acid to each well. The plates were read at 450 nm using a microplate reader (ThermoMax Molecular Devices, Sunnyvale, CA).

Sera obtained from convalescent pigs were used as reference positive serum. Sera from a 1-day-old colostrum-deprived piglet, and 11 specific pathogen free (SPF) pigs were used as reference negative sera. The specificity of this ELISA was examined

using sera from 60 healthy pigs that were naturally colonized with *H. parasuis* in the tonsils and had been experimentally infected with *A. pleuropneumoniae.*

Immunization and challenge of pigs

All animal experiments were carried out according to the guidelines of the Institutional Animal Care and Committee, University of Minnesota. The protective efficacy of a vaccine based on recombinant *H. parasuis* protein was evaluated by immunizing 11 weaned pigs by intramuscular injection of recombinant protein (200 μg) emulsified in oil-in-water adjuvant on day 0. Subsequent booster injections were administered on day 14 (Figure 2.1). Another group of 11 pigs was not vaccinated and served as positive control. The pigs were housed at the University of Minnesota animal research isolation units and were fed ad libitum and with free access to water.

Challenge was performed with the *H. parasuis* serotype 5 reference strain (Nagasaki), passed onto embryonated chicken eggs prior to challenge to increase the expression of capsule, a known virulent factor of Gram-negative bacteria (Oliveira and Pijoan, 2002). Vaccinated and non-vaccinated pigs were inoculated on day 34 of the experiment (20 days after the second dose of vaccine) with 1 ml of inoculum containing 10^{10} CFU *H. parasuis* administered intranasally. Pigs were observed daily for clinical signs of disease, such as fever (temperature ≥ 104ºF), respiratory distress, lameness, and/or CNS signs, and euthanized on day 41 of the experiment or when showing severe clinical signs of disease.

Vaccinated pigs were bled before vaccination and before challenge. Sera were separated by centrifugation and stored at -20°C. Sera were tested for antibodies

against recombinant *H. parasuis* protein using a commercially available *H. parasuis* antibody ELISA test kit (Biocheck, Scarborough, ME, USA) developed based on the recombinant protein ELISA previously described.

Statistical analysis

Serology data (OD values) were statistically analyzed for differences between *A. pleuropneumoniae* infected, SPF and convalescent pigs by Kruskal-Wallis test followed by Mann-Whitney pairwise comparison and Bonferroni correction ($p<0.016$). Fisher's exact test was used to compare mortality data and proportion of affected pigs between vaccinated and unvaccinated groups in the challenge experiment. Mann-Whitney U test was used to compare distribution of ELISA sample-to-positive (SP) ratio values between vaccinated and unvaccinated groups ($p<0.05$). ELISA SP values and body temperatures were compared within groups throughout the course of infection by repeated measures Friedman's ANOVA Test, followed by multiple pairwise comparisons using Wilcoxon signed rank test with Bonferroni correction, $p<0.016$ for SP values and $P<0.007$ for body temperatures.

Results

Bacterial strains, genotyping and serotyping analysis

All pigs treated with ceftiofur for Glasser's disease survived the infection. The diseased pigs that were necropsied presented lesions characteristic of *H. parasuis* infection, such as polyserositis and arthritis. Twenty-two *H. parasuis* isolates were obtained from eight of the 10 necropsied pigs. ERIC-PCR genomic fingerprints revealed seven different genotypes among the 20 isolates. Serotyping analysis

revealed that two out of the seven strains belonged to serovar 4 and the remaining strains were nontypeable. These seven strains were further analyzed by Western blot.

Detection and identification of a highly immunogenic species-specific protein in H. parasuis

Western blots performed with *H. parasuis* whole-cell lysate and convalescent sera from pigs in the *H. parasuis* field outbreak resulted in multiple bands ranging from 27 kDa to 77 kDa. One single band with an estimated molecular weight of 52 kDa, was consistently detected and clearly immunodominant in all Western blots using convalescent sera from each of the 10 pigs tested against each of the 7 genotypically distinct *H. parasuis* field outbreak strains (Figure 2.2) and 13 distinct *H. parasuis* reference strains (Figure 2.3). No bands were observed in Western blots that used the serum of piglets prior to suckling colostrum, indicating lack of non-specific reactions with porcine serum (Figure 2.4). Additionally, convalescent sera did not recognize this 52 KDa protein in any protein preparations from *P. multocida, B. bronchiseptica, A. pleuropneumoniae, A. idolicus, A. minus, A. suis, A. porcinus, E. coli, S. cholerasuis and S. suis* (Figure 2.5).

The 52-kDa protein from all seven outbreak strains and two reference strains (serotype 3, a non-virulent strain and serotype 5, a highly virulent strain) was subjected to N-terminal amino acid sequence analysis. The partial sequences obtained were blasted using NCBI database and the complete sequence was deduced by comparison with *H. parasuis* serovar 5 SH0165 genomic sequence (Yue et al., 2009). This protein was consistently identified as oligopeptide permease A (OppA) in the GenBank. The OppA gene codes for this protein, which is a transmembrane protein

responsible for transporting and internalizing 3-5 amino acid molecules in bacteria (Higgins and Hardie, 1983). The complete nucleotide and amino acid sequences for OppA had approximately 1584 base pairs and 527 amino acids, respectively. BLAST searching on GenBank for nucleotide sequences producing significant alignments matched *H. parasuis* SH0165 (CP001321.1) with 100% identity, and *H. parasuis* ZJ0906 (CP005384.1) with 99% identity. The subsequent closest match was *A. pleuropneumoniae* serovar 7 str.AP76 (CP001091.1) with 69% identity. BLAST searching of GenBank for non-redundant protein sequences revealed the highest amino acid sequence identity of 99% with *H. parasuis* OppA protein, followed by 51% identity with *Mannheimia granulomatis* (WP027074235.1), and by 49% identity with *A. pleuropneumoniae* (WP005608218.1).

In house ELISA analysis

The OppA ELISA was able to detect antibodies against *H. parasuis* OppA in sera from the 10 convalescent pigs with OD values ranging from 0.21 to 1.20. Levels of specific IgG antibodies against *H. parasuis* rOppA protein were statistically higher in convalescent pigs (mean OD 0.59) than in healthy pigs nasally colonized with *H. parasuis* (mean OD 0.07) or SPF pigs (mean OD 0.02) ($p<0.016$) (Figure 2.6). The colostrum-deprived piglet also had a very low antibody level (OD 0.02).

Recombinant OppA confers no protective immunity in a pig model

Piglets harbored *H. parasuis* in their noses at the beginning of the study, but tested negative for antibodies against OppA in serum by ELISA prior to inoculation. All pigs vaccinated with rOppA protein seroconverted two weeks after the second dose of

vaccine (SP ratio > 0.5) (Figure 2.7). Non-vaccinated pigs did not have antibodies against rOppA protein.

Challenged pigs had fever and mean rectal temperature reached the highest point for non-vaccinated and vaccinated pigs at 2 and 4 days post-infection (DPI), respectively (Figure 2.8). On 2 DPI, one pig from the vaccinated group was lethargic and hypothermic and it was euthanized. No gross lesions were observed. On 4 DPI, one non-vaccinated pig presented nervous clinical signs and two vaccinated pigs presented swollen joints and respiratory distress and were euthanized. Moderate amounts of turbid fluid and fibrin within pleural, pericardial and peritoneal cavities and joints were observed in these pigs. The remaining 18 pigs did not show severe clinical signs. They were euthanized 7 days post-challenge. The main lesion found in these pigs was pneumonia, observed in 5 out of 18 pigs (4 control and 1 vaccinated pigs). Only pigs that presented clinical signs and/or lesions at necropsy (four control and five vaccinated) tested positive for *H. parasuis* by PCR from swabs collected from systemic sites at necropsy. Nevertheless, there were no differences between vaccinated and non-vaccinated groups on clinical signs, lesions or mortality (Table 2.1, $p>0.05$).

Discussion

In this study, the OppA, a 52-kDa protein, was identified in *H. parasuis* by screening whole cell proteins from virulent strains using convalescent sera from pigs. This protein was highly immunogenic, specific to *H. parasuis* and found in multiple serotypes. Antibodies against OppA were found only in pigs infected systemically, and not in healthy pigs colonized with *H. parasuis* in the upper respiratory tract.

Therefore, detection of OppA antibodies can be used as a diagnostic marker of systemic infection.

The OppA protein, with a total length of 1584 bp, belongs to the ATP-binding cassette (ABC) transporter systems. These oligopeptide transport systems are composed of five subunits: OppA, OppB, OppC, OppD and OppF, OppA being the extracellular biding subunit, responsible for capturing substrates from the environment (Monet, 2003). Even though the exact localization of *H. parasuis* OppA protein is unknown, oligopeptide-binding proteins are usually localized in the periplasm of Gram-negative bacteria, the region between the plasma membrane and the outer membrane of the bacterial cell. These proteins utilize energy from ATP hydrolysis to transport a variety of substances across the membrane (Monnet, 2003). The role of OppA in the development of protective immunity is unclear since most immunogenic proteins in Gram-negative bacteria are associated with the outer membrane. However, immunization of mice with OppA resulted in a protective immune response against *Yersinia pestis* (Tanabe et al., 2006). Anti-OppA antibodies are also present in convalescent sera of human patients that have recovered from systemic infections with *Y. pestis* (Tanabe et al., 2006) and *Borrelia burgdorferi* (Nowalk et al., 2006). Specifically for *H. parasuis,* Hong et al (2011) identified OppA as a potentially immunogenic protein through an approach different than the one described in the present study. In that study, OppA was identified through reverse vaccinology and its immunogenicity was demonstrated in mice. In our study we confirmed the immunogenicity of this protein in the pig, *H. parasuis* natural host species. However, a strong anti-OppA immune response was not able to protect pigs against a challenge with virulent *H. parasuis*.

The purified, rOppA protein was further used to track anti-*H. parasuis* OppA antibodies in serum by ELISA. All convalescent pigs had antibodies against the *H. parasuis* rOppA antigen, while sera from a colostrum-deprived piglet, SPF pigs and from *A. pleuropneumoniae* infected, *H. parasuis* colonized pigs had undetectable to very low levels of antibody against rOppA (Figure 2.6). Specific binding of pig IgG antibodies against *H. parasuis* rOppA and lack of cross-reaction with IgG against *A. pleuropneumoniae* was clearly demonstrated. Interestingly, the *A. pleuropneumoniae* infected pigs used in this study were colonized with *H. parasuis* in the tonsils. Therefore, these results are indicative that pigs colonized with *H. parasuis,* and have not gone through a systemic infection, do not develop anti-OppA antibodies. In contrast, pigs that have survived a systemic infection are consistently positive for OppA antibodies. For that reason, *H. parasuis* OppA ELISA can be useful to track systemic infection against different serotypes and strains of *H. parasuis*. *H. parasuis* serologic profiles were recently assessed using the commercial *H. parasuis* OppA ELISA. The commercial *H. parasuis* OppA ELISA kit detected maternally derived antibodies following either *H. parasuis* vaccination with a live *H. parasuis* vaccine or autogenous killed vaccine (Galina Pantoja et al., 2014). In this study, healthy pigs colonized with *H. parasuis* but not infected, did not have antibodies against OppA, which is in agreement with our results.

Universal antigens such as OppA are highly desirable for vaccine development, especially regarding *H. parasuis*, a highly heterogeneous species (Oliveira et al., 2003b; Mullins et al., 2012; Boerlin et al., 2013).The robust antibody response to rOppA protein immunization shows that it is highly immunogenic in swine. However, OppA protein seroconversion did not translate to protection against *H. parasuis*

infection in the vaccine trial. Protective immunity to *H. parasuis* infection has been established to be mediated by antibodies (Martin de la Fuente et al., 2009a; Nedbalcova et al., 2011), but a *H. parasuis* antigen that is able to elicit complete protective immunity against all *H. parasuis* strains still has not been identified. In order to develop a more efficient subunit vaccine, a combination of antigens, each antigen inducing protection against a fraction of isolates, might be needed.

In summary, a highly immunogenic and species-specific protein in *H. parasuis* was identified by screening pig's convalescent sera for antibodies against *H. parasuis* specific proteins. OppA was further characterized by sequencing, cloning and purifying the immunogenic protein. We also demonstrated the diagnostic potential of *H. parasuis* OppA through the development of a species-specific serological test that can be used to characterize antibody profiles against *H. parasuis*. However, OppA did not show potential as a vaccine antigen since it was unable to elicit a protective immune response.

Table 2.1. Serological status and clinical outcome of intranasally inoculated pigs with *Haemophilus parasuis* with and without previous vaccination with OppA vaccine.

Group	Mean IgG SP ratio[a]	Number of pigs euthanized[b]	Number of pigs with gross lesions[c]
Non-vaccinated	0.00	1/11	4/11
Vaccinated	5.94	3/11	4/11

[a]Mean antibody response (SP ratio) of pigs on day 28 of the experiment

[b]Refers to pigs that had to be euthanized because of severe clinical signs. Pigs showing no severe clinical signs were euthanized at the scheduled necropsy day, day 41.

[c]Gross lesions observed at necropsy

Figure 2.1. Vaccination with recombinant recombinant OppA protein, challenge with *Haemophilus parasuis* and necropsy schedule.

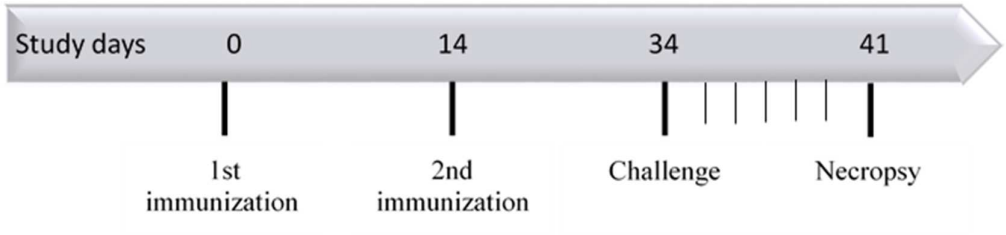


Figure 2.2. Demonstration of immunogenic proteins in 7 *Haemophilus parasuis* field strains obtained from brain (BR), pleura (PL), and pericardium (PC) and 1 swine related bacterium species *Actinobacillus indolicus* (AI) by Western blot with convalescent serum from a pig that survived a *H. parasuis* systemic infection.

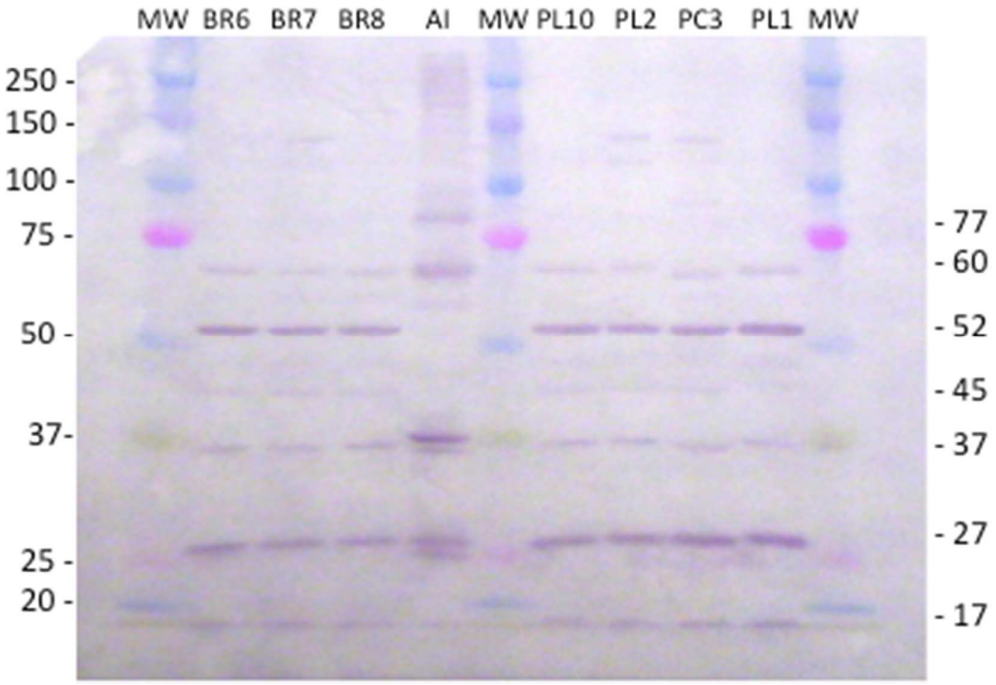


Figure 2.3. Detection of an immunogenic protein in 13 *Haemophilus parasuis* reference strains by Western blot with convalescent serum from a pig that survived a *H. parasuis* systemic infection. Serotypes are indicated in each lane.

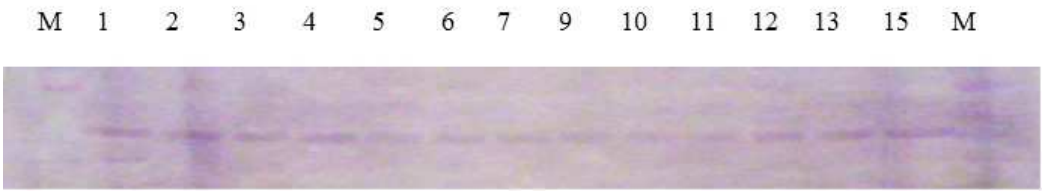


Figure 2.4. No non-specific detections by Western blot were observed. *Haemophilus parasuis* field strains obtained from brain (BR), pleura (PL), and pericardium (PC) and 3 reference strains (serotypes 3, 5, and 12).

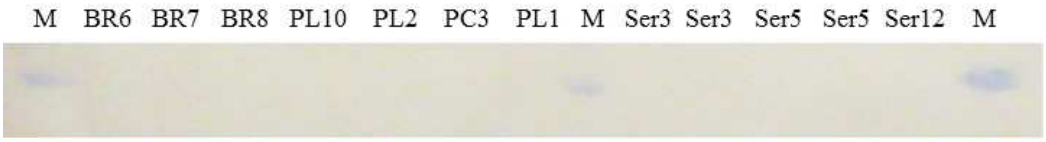


Figure 2.5. Specificity testing by Western blot using serum from a convalescent pig that survived a *Haemophilus parasuis* infection. An immunogenic protein was detected in a *H. parasuis* strain isolated from brain (BR7) and in the reference strain for serotype 5 (Ser 5). No immunogenic proteins were detected in *Actinobacillus porcinus* (AP), *Actinobacillus indolicus* (AI), *Actinobacillus minor* (AM), *Actinobacillus pleuropneumoniae* (APP), *Actinobacillus suis* (AS), *Streptococcus suis* (SS), *Bordetella bronchiseptica* (BB), *Pasteurella multocida* (PM), *Escherichia coli (EC), Salmonella choleraesuis* (SC).

Figure 2.6. Mean OD ELISA values against *Haemophilus parasuis* recombinant OppA in serum (Mean ± 1 SE). *Actinobaciluus pleuropneumoniae* (App) infected pigs, positive for *H. parasuis* in nasal cavity (colonized) (group 1, n=80), SPF pigs (group 2, n=11) and *H. parasuis* convalescent pigs (Group 3, n=10). Different letters represent statistical differences between groups ($p<0.01$).

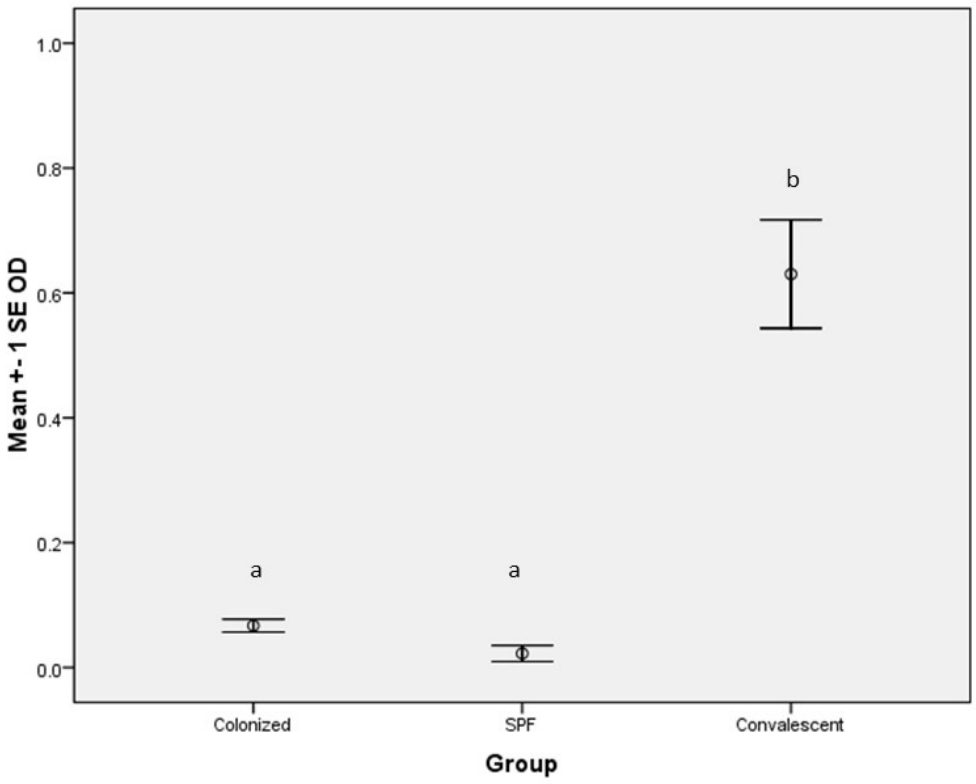


Figure 2.7. Recombinant OppA-specific antibodies measured by ELISA in weaned pigs. Pigs were vaccinated with rOppA subunit vaccine on days 0 and 14 of the study. Results show average SP ratio +/- 1 standard error (SE). *Indicates significant differences between groups (P<0.01).

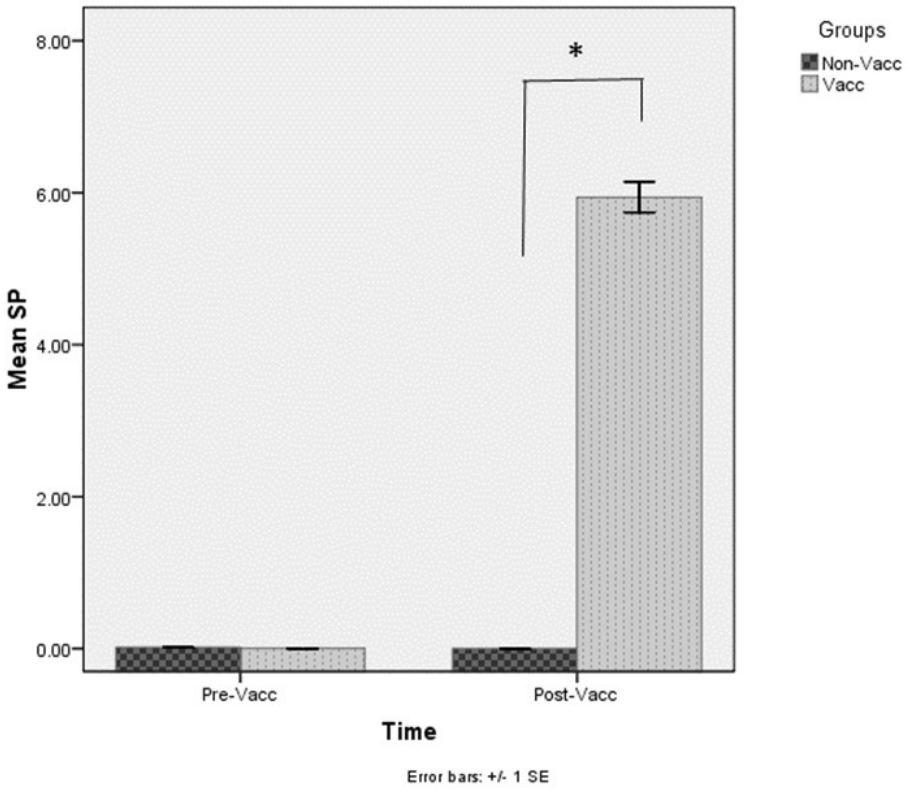
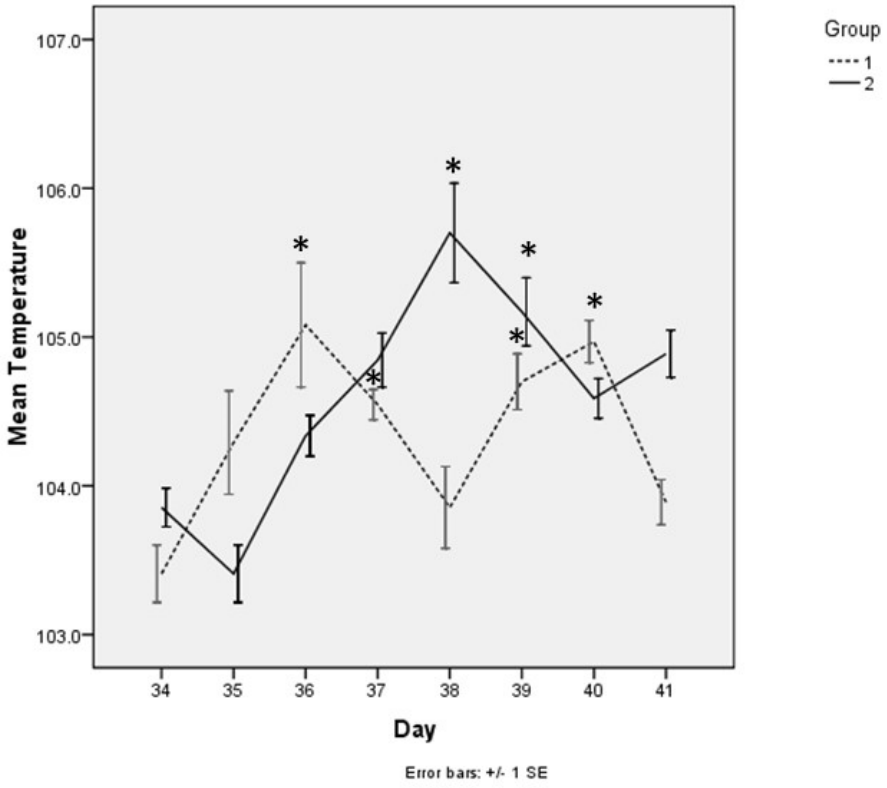


Figure 2.8. Body temperature (mean ± standard error) measured every day after challenge for group 1 (non-vaccinated) and group 2 (vaccinated). *Indicates significant differences within groups compared to baseline (day 34) (P<0.007).



CHAPTER 3

EXPERIMENTAL COLONIZATION OF CONVENTIONAL PIGS WITH PATHOGENIC *HAEMOPHILUS PARASUIS*

Macedo N, Rovira A, Torremorell M. Experimental colonization of conventional pigs with pathogenic *Haemophilus parasuis*. [In preparation]

Abstract

Glasser's disease is a swine illness caused by the bacterium *Haemophilus parasuis*. Pathogenic strains of *H. parasuis* colonize the upper respiratory tract (URT) of healthy pigs and, under certain conditions, can spread systemically and cause Glasser's disease. Experimental inoculation models described for *H. parasuis* are designed to develop the disease and not to create an asymptomatic colonization of the URT. Therefore, the primary objective of this study was to develop an experimental model of pathogenic *H. parasuis* infection that mimics URT colonization in conventional pigs. Sixteen conventional weaned pigs were divided into 3 groups. At day 0 of the study, pigs in groups 1 and 2 (n=6 each) received 10^4 or 10^6 CFU of highly virulent *H. parasuis* strain Nagasaki intranasally, and group 3 (n=4) received saline. Clinical evaluations and bacterial isolation from nasal swabs were performed daily and all *H. parasuis* isolates obtained were characterized by ERIC-PCR genotyping. ERIC-PCR genotyping demonstrated that pigs carried a commensal *H. parasuis* strain. This strain was frequently isolated before and after inoculation from the nose of all the pigs throughout the study. The Nagasaki strain, also identified by ERIC-PCR, was recovered from the nose of five pigs after inoculation and from tracheal swabs from ten pigs collected at necropsy. Overall, the Nagasaki strain was isolated at least once from the URT of all 12 inoculated pigs, but was never recovered from systemic sites, nor control pigs or before inoculation. There were no differences in isolation of the Nagasaki strain based on inoculation dose. No fever or clinical signs were observed in any of the pigs throughout the study. The absence of fever, clinical signs, lesions and bacteremia demonstrated that there was no systemic infection. In summary, the inoculation model described reproduced URT colonization with a pathogenic *H. parasuis* strain without causing systemic disease. This model

should prove useful to study factors that mediate *H. parasuis* colonization and expression of Glasser's disease.

Introduction

Glasser's disease is a serious swine illness caused by the Gram-negative bacterium *Haemophilus parasuis*. Glasser's disease is characterized by fibrinous polyserositis, arthritis and meningitis (Oliveira and Pijoan, 2004). Numerous strains of *H. parasuis* have been described through genotypic and phenotypic methods, some of which are pathogenic, while others are not (Oliveira et al., 2003, Olvera et al., 2006). Most conventional pigs harbor non-pathogenic *H. parasuis* in their noses (Amano et al., 1994; Vahle et al., 1997), tonsils (Macedo et al., 2014) and trachea (Segales et al., 1997). However, pathogenic strains also colonize the upper respiratory tract (URT) of healthy pigs (Oliveira et al., 2003a) and, under certain conditions, can spread systemically and cause Glasser's disease. The conditions that favor the development of disease are currently unknown. Several hypotheses have been suggested such as infection with a new strain when maternal antibodies wane or are no longer present (Pijoan et al., 1997, Blanco et al., 2004), co-infection with other pathogens (Yu et al., 2012), and certain uses of antibiotics (Macedo et al., 2014). Because of the complex nature of this problem, these hypotheses can only be tested by using an experimental model that can reproduce colonization of the URT with pathogenic *H. parasuis* under controlled conditions.

Numerous experimental inoculation models have been described for *H. parasuis*. However, they are all designed to develop the disease and not to create an asymptomatic colonization of the URT. In addition, most of these models use

cesarean-derived colostrum-deprived (CDCD) (Vahle et al., 1995; 1997), naturally-farrowed artificially-reared (Oliveira et al., 2003a) or specific pathogen free (Nielsen, 1993; Amano et al., 1996; 1997) pigs, which may not fully represent the conventional pig in terms of the immune response and the bacteriological nasal flora. Therefore, the primary objective of this study was to develop an experimental model of pathogenic *H. parasuis* that mimics nasal colonization in conventional pigs. The development of a pig model that preserves the status of conventional pigs will be a valuable tool to study *H. parasuis* colonization and development of disease.

Materials and Methods

Experimental animals

Sixteen 3-week-old conventional pigs were selected from a commercial swine herd. This herd was free from significant swine pathogens, including influenza virus, porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*, and had no history of Glasser's disease. The pigs were housed at the University of Minnesota animal isolation facility (Saint Paul, MN, USA) with each group placed in a separate isolation room. The pigs were identified and assigned to three groups of six or four pigs each. Pigs received feed and water ad libitum throughout the study. All pigs were monitored daily and cared for according to the University of Minnesota approved IACUC (Institutional Animal Care and Use Committee) protocols.

Bacterial strain

The *H. parasuis* Nagasaki strain was selected for the inoculation of piglets because of its known virulent properties. This strain has been extensively described in the

literature as highly virulent for its ability to cause polyserositis and sudden death in pigs (Amano et al., 1997; Martin de la Fuente et al., 2009a; Frandoloso et al., 2011; Olvera et al., 2011), invade endothelial (Vanier et al., 2006; Aragon et al., 2010; Frandoloso et al., 2013) and epithelial (Frandoloso et al., 2012b) cells in vitro, survive phagocytosis (Olvera et al., 2009), be resistant to serum (Cerda-Cuellar et al., 2008) and possess the virulent group 1 vtaA gene (Olvera et al., 2010).

Before inoculation, Nagasaki strain was passaged in chicken eggs according to Oliveira and Pijoan (2002). Briefly, Nagasaki strain was grown on chocolate agar and incubated at 37°C in a 5% CO_2 atmosphere for 18 hours. Bacterial growth was harvested from plates and suspended in sterile PBS. Ten-day old embryonated chicken eggs were inoculated through the chorio-allantoid membrane (CAM) using 0.2 ml of a bacterial suspension containing 10^9 colony-forming units (CFU)/ml of bacteria. Inoculated eggs were incubated at 37°C for 72 hours. Following the incubation period, eggs were opened and swabs were taken from the CAM. Swabs were cultured on blood agar with a *Staphylococcus aureus* streak in order to check the purity of the inoculum and then on chocolate agar. Bacterial growth was harvested from chocolate agar plates and frozen at -80°C until use. One tube from each culture was thawed and a standard bacterial count was performed. Original cultures were diluted to produce inocula containing 10^4 and 10^6 CFU/ml.

Inoculation of piglets

Pigs were inoculated with 1 mL of 10^4 CFU/ml of Nagasaki strain inoculum (low dose group (LD), n=6), 1.0 mL of 10^6 CFU/ml of Nagasaki strain inoculum (high dose group (HD), n=6), or 1 mL of sterile saline (control group, n=4) at day 0 of the study

(Figure 3.1). Inoculum was administered intranasally (0.5 mL in each nostril). Before the inoculation, all pigs were sedated by an intramuscular injection in the cervical muscles of a dissociative anesthetic at the recommended dose of 6.6 mg/kg (0.06 ml/kg) (Telazol®, Fort Dodge Animal Health, Fort Dodge, IA). Once sedation was achieved (1-2 minutes), pigs were held upright and the inoculum was instilled slowly into each nostril using a 1 mL syringe.

Clinical-pathological evaluation and sample collection

Rectal temperature and clinical signs compatible with Glässer's disease (cough, lethargy, abdominal breath, lameness and nervous signs) were recorded daily after bacterial inoculation (day 0). Blood samples were collected at 1, 3 and 4 days post-inoculation (dpi) to monitor bacteremia by bacterial isolation and PCR. Half of the pigs in each group were randomly selected and euthanized at 4 dpi. The remaining pigs were euthanized at 7 dpi (Figure 3.1). All pigs were necropsied for evaluation of gross lesions with special focus on those potentially attributable to *H. parasuis* infection (Oliveira and Pijoan, 2004). Samples of lung, liver, spleen and kidney were collected at necropsy and evaluated for histological lesions.

Nasal swabs were taken before inoculation and daily post-inoculation and assayed for the presence of *H. parasuis* by bacterial isolation. Swabs of nasal cavity, tonsil, trachea and serosas (same swab was rubbed on pleura, pericardium, peritoneum and joint) were collected postmortem and assayed for the presence of *H. parasuis* by bacterial isolation and PCR (Oliveira et al., 2001b). Additionally, *H. parasuis* isolation was attempted from lung, liver, spleen and kidney samples collected at necropsy.

Serum samples collected before inoculation and at necropsy were tested for *H. parasuis* antibodies using the BioChek *Haemophilus parasuis* Oligopeptide permease A (OppA) Antibody Test Kit (BioChek, Scarborough, ME), which is based on the *H. parasuis* species-specific protein OppA (chapter 2). Samples with sample to positive ratios (S/P) > 0.5 were considered positive.

Isolation and identification of H. parasuis

For isolation of *H. parasuis*, swabs were plated onto sheep blood agar streaked with a nurse *Staphylococcus aureus*, cultured and incubated at 37°C in a 5% carbon dioxide (CO2) atmosphere for 24 to 48 hours. *H. parasuis*-like colonies were selected for biochemical identification performed as previously described (Oliveira et al., 2001b).

In order to differentiate strain Nagasaki from any other *H. parasuis* strains that the pigs naturally carried, *H. parasuis* isolates were genotyped by ERIC-PCR (Rafiee et al., 2000). *H. parasuis* isolates were also tested using a multiplex PCR to detect *H. parasuis* group 1 virulence associated trimeric autotransporter (vtaA) gene (Olvera et al., 2012).

Statistical analysis

The proportion of pigs positive based on Nagasaki strain isolation from nose and trachea and the proportion of pigs presenting clinical signs of disease including fever (rectal temperature above 40°C) were compared by Fisher's exact test.

Results

H. parasuis colonization

Prior to inoculation of the Nagasaki strain, nasal swab cultures yielded *H. parasuis* isolates from 14 out of 16 pigs (Table 3.1). These 14 isolates had identical ERIC-PCR fingerprint, and were therefore considered one strain, denominated "farm" strain henceforward (Figure 3.2A). There were 53 additional isolations of the *H. parasuis* farm strain throughout the study (Table 3.1). After inoculation, the Nagasaki strain was first isolated from three different pigs belonging to the LD group at 1, 3 and 5 dpi and from one pig from the HD group at 3 dpi (Table 3.1). Nagasaki strain was not isolated from control pigs. At necropsy, the Nagasaki strain was recovered from tracheal swabs from 11 inoculated pigs, while tonsillar swabs yielded only one isolate (Table 3.1). The number of pigs colonized by the Nagasaki strain was significantly higher in LD and HD groups when compared to the control group ($p=0.005$). All pigs tested positive by PCR for detection of *H. parasuis* at the species levels in the nose at necropsy, while 15 out of 16 tested positive in the tonsils and half of the pigs tested positive in the trachea (Table 3.2).

Absence of H. parasuis systemic infection

After inoculation, pigs did not have any clinical signs of disease. At necropsy, no gross or microscopic lesions were observed. *H. parasuis* was not isolated from blood collected at 1, 3 and 4 dpi or from tissues (liver, spleen, kidney and lung) or serosal swabs collected at necropsy from any of the pigs. Serosal swabs also tested negative for *H. parasuis* by PCR (Table 3.2). Antibodies against *H. parasuis* antigen OppA were not detected before inoculation, or at 4 and 7 dpi.

Discussion

This study showed that conventional pigs can be used as a model to study mucosal colonization with a *H. parasuis* pathogenic strain. *H. parasuis* frequently colonizes the surface of the mucosa of the swine respiratory tract and colonization is considered an important pre-requisite for subsequent invasion of host tissues and progression to Glasser's disease (Vahle et al., 1995). However, better understanding of *H. parasuis* disease mechanisms and host immune responses is required. To this end, we have established a model of *H. parasuis* colonization by a pathogenic strain in the absence of systemic disease in conventional pigs.

Successful nasal colonization was demonstrated by isolation of the Nagasaki strain at least once from the URT of all 12 inoculated pigs between days 1 and 7 post-inoculation. On the other hand, the absence of systemic spread was evidenced by lack of clinical signs, lack of recovery of Nagasaki strain from blood or systemic tissues, and lack of gross or microscopic lesions at necropsy.

Because the ideal inoculation dose was previously unknown, two different doses were used. There were no significant differences on the number of Nagasaki isolates obtained based on inoculation dose. Nagasaki strain has been used before to inoculate CDCD pigs at a low dose of 5 x 10^4 CFU/mL without causing disease, however evidence of colonization was not provided in that study (Martin de la Fuente et al., 2009a). In a different study, inoculation of pigs fed bovine colostrum and milk replacer with Nagasaki strain at 10^4 and 10^6 CFU resulted in systemic disease (Oliveira et al., 2003a). This is the first study to report the use of low dose Nagasaki strain to colonize conventional pigs.

Therefore, under the conditions of the present study, doses between 10^4 and 10^6 CFU/mL were low enough not to cause systemic infection as well as high enough for nasal and tracheal colonization of conventional weaned pigs.

H. parasuis isolation from tonsils was not common in this study, which is in agreement with other studies showing that the nasal cavity might be the primary site for *H. parasuis* respiratory mucosal colonization (Vahle et al., 1995; 1997). However, when tonsil swabs were tested by PCR, *H. parasuis* DNA detection was common (Table 3.2). Tracheal swabs yielded higher numbers of Nagasaki isolates than the nasal swabs. Previous reports have described the recovery of *H. parasuis* from trachea of CDCD pigs intranasally inoculated, even though *H. parasuis* was isolated more frequently from nasal cavity (Vahle et al., 1995; 1997). In this study, the *H. parasuis* farm and Nagasaki strains seemed to have a predilection for nasal and tracheal mucosa, respectively. Alternatively, the farm strain found naturally in the nose may have prevented the Nagasaki strain from stablishing itself in the same environment. Additionally, in conventional pigs, trachea might represent a less competitive niche for *H. parasuis* colonization, which may explain why tracheal swabs yielded higher number of Nagasaki isolates compared to nasal swabs.

No rise on antibody levels was detected in inoculated or control pigs during the study period. This supports the conclusion that pigs were not systemically infected. The *H. parasuis* OppA ELISA test detects IgG antibodies in serum usually 7 to 14 days after pigs are systemically infected or vaccinated (chapter 2). Therefore, 7 days may have not been enough time to observe seroconversion. However, euthanasia times of 4 and 7 dpi were selected to maximize the chances of isolating *H. parasuis* from systemic

samples and observing lesions in systemic tissues. Further investigations on other immune parameters, such as IgA antibodies and cellular responses, are needed since they might play a role on limiting systemic infection.

In summary, in contrast to *H. parasuis*-free pig models, the colonization model using conventional pigs mimics the status of commercial animals. Pathogenic strains of *H. parasuis* can colonize the nose and trachea of conventional pigs for at least 7 days. Mechanisms by which pathogenic *H. parasuis* strains compete with many other microorganisms for colonization sites in the URT and invade systemic tissues are not well understood. This model should prove useful to study factors that mediate *H. parasuis* colonization and expression of Glasser's disease.

Table 3.1. Summary of type of *Haemophilus parasuis* isolates obtained from weaned pigs throughout the study.

			Days post-inoculation						
Group (inoculum dose)	Pig #	Pre-inoc.	1	2	3	4	5	6	7
10^4 CFU/mL	986	Farm[1,2]	-[3]	-	-	**NAG[5] NAG/Trachea**	†[6]	†	†
	976	Farm	NAG[4]	Farm	Farm	**NAG/Trachea**	†	†	†
	979	Farm	-	Farm	-	Farm **NAG/Trachea**	†	†	†
	980	Farm	Farm	Farm	NAG	Farm	Farm	Farm	**NAG/Tr**
	975	-	-	Farm	-	Farm	NAG	Farm	**NAG/Tr**
	987	Farm	Farm	Farm	Farm			Farm	**NAG/Tr**
10^6 CFU/mL	974	-	Farm	Farm	-	**Farm NAG/Trachea**	†	†	†
	978	Farm	Farm	Farm	Farm	**Farm NAG/Trachea**	†	†	†
	989	Farm	Farm	-	Farm	**Farm NAG/Trachea**	†	†	†
	983	Farm	-	Farm	NAG	Farm	Farm		**Farm NAG/Tr**
	984	Farm	-		-	Farm	Farm		**NAG/Tc**
	988	Farm	Farm	Farm	-	Farm	Farm	Farm	**Farm NAG/Tr**
Control (PBS)	977	Farm	-	Farm	Farm	-	†	†	†
	985	Farm	-	-	-	-	†	†	†
	981	Farm	-	Farm	Farm	Farm	Farm	Farm	-
	982	Farm	Farm	Farm	Farm	Farm	Farm	Farm	-

[1] Farm strain

[2] *H. parasuis* isolate recovered from nasal swab unless otherwise specified

[3] No *H. parasuis* isolation

[4] NAG: Nagasaki strain

[5] Bold specifies isolate recovered at necropsy

[6] Pig euthanized on 4 dpi.

Table 3.2. *Haemophilus parasuis* PCR results from swabs collected from pigs at necropsy 4 or 7 days after inoculation with *H. parasuis*.

			Swabs collected at necropsy			
Group (inoculum dose)	Necropsy day	Pig #	Nose	Tonsil	Trachea	Serosas
Lower dose (10^4 CFU/mL)	4	986	+	+	+	-
		976	+	+	+	-
		979	+	+	-	-
	7	980	+	+	-	-
		975	+	-	-	-
		987	+	+	+	-
Higher dose (10^6 CFU/mL)	4	974	+	+	+	-
		978	+	+	-	-
		989	+	+	+	-
	7	983	+	+	-	-
		984	+	+	+	-
		988	+	+	+	-
Control (PBS)	4	977	+	+	-	-
		985	+	+	-	-
	7	981	+	+	-	-
		982	+	+	+	-

Figure 3.1. *Haemophilus parasuis* colonization model: Experimental design and sampling times. [a]Pigs were inoculated with 1.0 mL of 10^4 CFU/ml or 10^6 CFU/ml of Nagasaki strain or 1.0 mL of sterile saline intranasally at day 0 of the study.

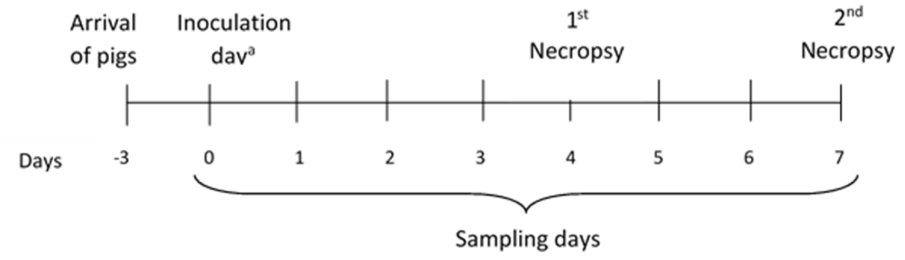
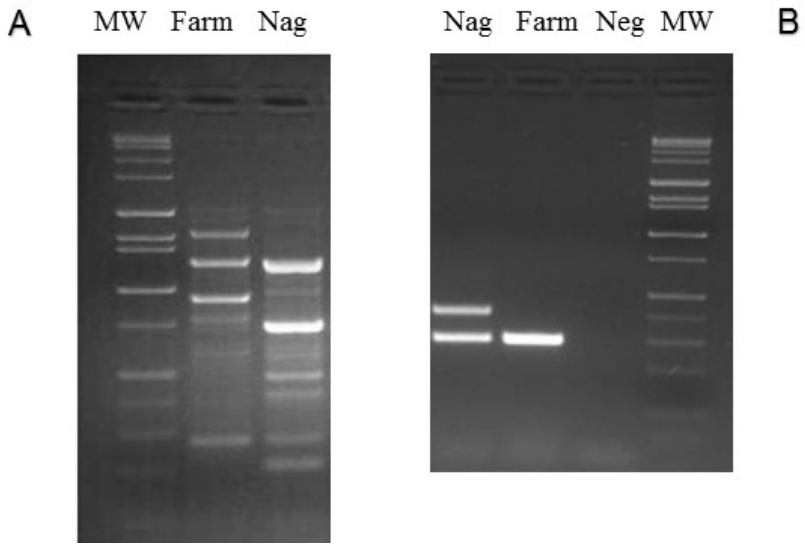


Figure 3.2. ERIC-PCR fingerprint profiles of *H. parasuis* strains in this study (A), and results of vtaA Multiplex PCR (B). MW: Molecular weight marker. Farm: farm strain. Nag: Nagasaki strain. Neg: Negative control.



CHAPTER 4

EFFECT OF ENROFLOXACIN ON THE CARRIER STAGE OF *HAEMOPHILUS PARASUIS* IN NATURALLY COLONIZED PIGS

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Abstract

The purpose of this study was to determine the effect of enrofloxacin in the carrier stage of *Haemophilus parasuis* in naturally colonized weaned pigs. Twenty-three pigs colonized by *H. parasuis* received either 7.5 mg/Kg of enrofloxacin or saline solution intramuscularly at weaning. Nasal and tonsillar swab samples were collected daily throughout the study and at necropsy and tested by quantitative PCR (qPCR). The *H. parasuis* isolates obtained from samples collected at necropsy were subjected to genotyping by enterobacterial repetitive intergenic consensus (ERIC)-PCR, and a multiplex PCR for the detection of the *vtaA* virulence associated trimeric autotransporter genes. *H. parasuis* was detected in the nasal cavity and tonsils of pigs in the control group throughout the study. Antibiotic treated pigs tested *H. parasuis* negative at 1 day post treatment (DPT), and the proportion of nasal samples that tested positive was statistically higher for control pigs compared with treated pigs at 1, 2, 3, 4, 5, 6 and 7 DPT, and at 2, 4 and 5 DPT for tonsil samples (p value < 0.003). Genotyping by ERIC-PCR demonstrated that pigs were colonized with a common *H. parasuis* strain at the end of the study. Isolates were negative for the *vtaA* gene, which indicated that they did not have the *vtaA* virulence factor. In conclusion, enrofloxacin significantly reduced the *H. parasuis* load in naturally colonized pigs, but was unable to eliminate the organism.

Introduction

Haemophilus parasuis is an economically significant Gram-negative organism, which colonizes the upper respiratory tract of pigs soon after birth (Smart et al., 1988; Oliveira et al., 2001a). The presence of humoral immunity generally prevents pigs from developing systemic disease (Solano-Aguilar et al., 1999; Cerda-Cuellar et al., 2010), which is commonly characterized by fibrinous polyserositis, arthritis and meningitis (Amano et al, 1994). Stress conditions coinciding with decay of maternal immunity, such as weaning and transport (Kirkwood et al., 2001), and co-infections with immunosuppressive agents, such as porcine reproductive and respiratory syndrome (PRRS) virus (Solano et al., 1998), have been suggested as risk factors for systemic invasion of *H. parasuis.*

Alterations in the carrier stage of *H. parasuis* at a young age have also been associated with the development of Glasser's disease during the post-weaning period (Pijoan et al., 1997). Most of the studies have focused on the effect of early weaning in the disruption of the colonization patterns under the presence of maternal immunity. In these studies, *H. parasuis* disease was exacerbated when pigs were colonized late when maternal immunity was waning (Oliveira et al., 2001; Cerda-Cuellar et al., 2010; Blanco et al., 2004). However, there is limited information on what other factors may alter the carrier state of *H. parasuis*.

Antibiotics are commonly used to mitigate the effects of bacterial disease in pigs in order to limit bacterial challenge. A recent study has reported that marbofloxacin treatment was able to reduce the nasal carriage of *H. parasuis* in weaned pigs (Vilalta et al., 2012). Another fluoroquinolone, enrofloxacin, is a common antimicrobial used

in farms to treat Glasser's disease in North America. Enrofloxacin is among the approved products by the US Food and Drug Administration Center of Veterinary Medicine for treatment and control of disease associated with *H. parasuis.* There is no information on what effect enrofloxacin may have specifically on the carrier state of *H. parasuis* in naturally colonized pigs and whether the carrier state is affected at all. Thus, the purpose of this study was to evaluate the effect of enrofloxacin in reducing *H. parasuis* colonization in weaned pigs.

Material and Methods

Animals and animal housing

Forty five, 1-week-old pigs with a history of Glasser's disease, porcine reproductive and respiratory syndrome (PRRS) virus, porcine circovirus type 2 (PCV2), and *Mycoplasma hyopneumoniae* were identified on a conventional North American farm and screened for the presence of *H. parasuis* in the upper respiratory tract using 16S ribosomal ribonucleic acid (rRNA) gene polymerase chain reaction (PCR) (Oliveira et al., 2001b). The pigs received PCV2 vaccine at 4 days of age and at weaning and *M. hyopneumoniae* vaccine at weaning. Of those 45 pigs, twenty-four 3-week-old weaned pigs that tested positive for *H. parasuis* were selected and moved to the University of Minnesota research isolation facility. Pigs were randomly divided into treatment (n = 12 pigs) and control groups (n = 12 pigs) and housed in two separated isolation rooms (1 pig in the control group died shortly after arrival due to an unrelated *H. parasuis* cause). The pigs were fed *ad libitum* and had free access to water. Pigs were cared for according to the guidelines of the Institutional Animal Care and Use Committee of the University of Minnesota.

Experimental design

On the day of arrival at the research facility, blood samples and nasal and tonsil swabs were collected from all pigs and tested by 16S rRNA gene PCR (Oliveira et al., 2001b) and qPCR as described in the next section. Pigs in the treatment group were treated with a single dose of injectable enrofloxacin (7.5 mg/kg BW Baytril; Bayer Animal Health, Shawnee, Kansas, USA) at 24 h post-arrival and those in the control group received saline solution intramuscularly. Tonsillar and nasal swabs were collected daily. At 3, 7, and 14 d post-treatment, four pigs from each group were randomly chosen and euthanized. At necropsy, blood samples and swabs from the nasal cavity, interior of the tonsil, trachea, lung, and peritoneal and pleural serosa were collected in duplicate. One swab from each organ was used for bacterial isolation and the other swab was tested by quantitative PCR (qPCR) as outlined in the next section. *H. parasuis* isolates were further characterized by ERIC-PCR (Rafiee et al., 2000) and virulence-associated trimeric autotransporter polymerase chain reaction (*vtaA*-PCR) (Olvera et al., 2012).

Quantitative PCR

Deoxyribonucleic acid (DNA) from swabs was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and then tested individually by qPCR (Turni et al., 2010), with modifications. Briefly, primers forward (59-CGACTTACTTGAAGCCATTCTTCTT-39) and reverse (59-CCGCTTGCCATACCCTCTT-39) were based on the *infB* gene of *H. parasuis*, which is also considered to be a genetic marker for phylogenetic studies of species, separating *H. parasuis* from other closely related species. The FAM-labelled *Taq*Man probe with a TAMRA quencher (59-6FAM-

ATCGGAAGTATTAGAATTAAGTGCTAMRA-39) was supplied by Applied Biosystems (Carlsbad, California, USA). The 25-mL reaction mix consisted of 7 mL of H2O, 10 mL of master mix, 100 nmol/L of CTinfF1, 400 nmol/L of reverse primer, 100 nmol/L of forward primer, and 5 mL of template. A standard curve was established by a 10-fold serial dilution of known quantities of the *H. parasuis* reference strain Nagasaki extracted DNA. The 7500 Fast System SDS software (Applied Biosystems) was used to calculate the quantity of unknown target sequences from the standard curve for the detector of that target. The reaction was run with the following cycling conditions: first cycle at 95°C for 2 min, followed by 30 cycles of 95°C for 20 s and 58°C for 60 s, followed by 1 cycle at 28°C for 1 min. All reactions, including the standard curve, were run in duplicate. Results are shown in CFU/reaction.

H. parasuis isolation and characterization

For isolation of *H. parasuis,* samples were plated onto sheep blood agar streaked with a nurse *Staphylococcus* sp. strain, cultured and incubated at 37ºC in a 5% carbon dioxide (CO_2) atmosphere for 24 - 48 hours. Up to 5 *H. parasuis*-like colonies per agar plate were selected for further identification (Oliveira et al., 2001b). Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) (Rafiee et al., 2000) was used to better characterize *H. parasuis* isolates.

A multiplex PCR based on *vtaA* genes (Olvera et al., 2012) was used to differentiate the non-virulent from potentially virulent *H. parasuis* isolates. *H. parasuis* possesses virulence associated trimeric autotransporters (*vtaA*): group 1 *vtaA* has been

associated with virulent strains; while group 3 *vtaA* gene is highly conserved among *H., parasuis* strains (Pina et al., 2009).

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were tested for *H. parasuis* antibodies using an enzyme-linked immunosorbent assay (ELISA) based on the *H. parasuis* species-specific protein OppA (chapter 2), using the Swine SK104 *Haemophilus parasuis* (OppA) Antibody Test Kit (BioChek, Scarborough, Maine). Samples testing sample to positive ratio (S/P) > 0.5 were considered positive.

Statistical analysis

Differences between the proportions of *H. parasuis*-positive pigs in treated *versus* control groups at each sampling time point were calculated using Fisher's exact probability test. The Bonferroni correction was used to address multiple comparisons ($\alpha = 0.003$).

Results

Polymerase chain reaction (PCR) targeting the 16S rRNA PCR was used to screen naturally colonized pigs at the farm of origin and to confirm their *H. parasuis* colonization status when the pigs arrived at the research facility. Tonsil and nasal swabs from all pigs tested positive for *H. parasuis* by 16 rRNA PCR on the day of arrival at the research facilities. No clinical signs of *H. parasuis* disease (fever, anorexia, lameness, thumping) were observed during the experiment.

Quantitative PCR was used to detect and quantify *H. parasuis* in the tonsil and nasal cavity during the study period. Before treatment, 22 out of 23 pigs (95.7%) tested positive by qPCR from tonsil swabs, while only 11 pigs (48%) tested positive from nasal swabs (Figures 4.1 and 4.2). Among the pigs that tested positive before treatment, the average *H. parasuis* load detected in tonsils and nasal cavities was 2.8 x 10^5 and 2.3 x 10^4 colony-forming units (CFUs)/reaction, respectively. Pigs in the control group tested positive throughout most of the study, while all treated pigs tested *H. parasuis* negative by qPCR at 1 day post-treatment (DPT) (Figures 4.1 and 4.2, Table 4.1). The proportion of nasal samples that tested positive was statistically higher for control pigs compared with treated pigs at 1, 2, 3, 4, 5, 6 and 7 DPT, and at 2, 4 and 5 DPT for tonsil samples (p value < 0.003) (Figures 4.1 and 4.2).

At necropsy, no gross lesions were observed and *H. parasuis* was detected by qPCR in 9 out of 11 control pigs in at least 1 of the 5 samples tested (Table 4.2). In contrast, only 4 out of 12 pigs in the treatment group tested positive. Interestingly, all 4 *H. parasuis* isolates obtained were recovered from samples collected at necropsy at 15 DPT, but not in the necropsies prior to that. ERIC-PCR profiles were obtained from the four isolates. One single cluster could be distinguished for the four isolates based on coverage of 90% agreement (Oliveira et al., 2003b).

The putative virulence factor, the group 1 *vtaA* gene, was not detected in any of the four isolates obtained, while the group 3 *vtaA,* highly conserved among *H. parasuis* strains, was detected in all isolates.

All anti-OppA serum antibody levels, as measured by ELISA S/P on arrival (mean S/P = 0.01) and at necropsy (mean S/P control pigs 0.001 *versus* mean S/P treated pigs 0.03) were very low and under the cut-off value of 0.5. All values were considered negative.

Discussion

In this study, we demonstrated that enrofloxacin reduced the number of *H. parasuis* in the tonsil and nasal cavity of pigs and decreased the number of pigs that were positive for *H. parasuis* by qPCR during the first week after treatment.

The presence of *H. parasuis* was demonstrated by gel-based PCR in samples from tonsils and nasal cavity in all pigs before treatment, while qPCR detected 95.7% positives on tonsils but only 48% positives in nasal cavity. Differences in results might be due to the lower sensitivity of the qPCR (4 x 10^3 CFU/mL) compared with the gel-based PCR (1 x 10^2 CFU/mL) (Oliveira et al., 2001b). However, qPCR was preferred in order to quantify the effect of the antibiotic on bacterial load. The fact that the pigs tested negative after treatment and that some pigs then became positive again suggests that the treatment decreased the level of *H. parasuis* below the threshold of qPCR detection, but did not completely eliminate *H. parasuis.*

These results are in agreement with a study investigating the effect of marbofloxacin in the *H. parasuis* carrier state in pigs at the farm level (Vilalta et al., 2012). Both marbofloxacin as well as enrofloxacin in the present study showed a similar effectiveness after treatment, reducing the amount of *H. parasuis* in the nasal cavity of pigs, but not eliminating it.

The rapid and relatively long effect of enrofloxacin on *H. parasuis* in the nasal cavity and tonsil of pigs can be attributed to enrofloxacin's tissue penetration, extended half-life, and rapid bactericidal effect in a concentration-dependent manner and prolonged post-antibiotic effect (Walker et al., 2006). Possible explanations for the antibiotic's inability to fully eliminate *H. parasuis* are that the dose of antibiotic may have not reached an adequate concentration at the colonization sites or that enrofloxacin induced the emergence of resistant strains. Even though the concentration of enrofloxacin in the nasal cavity or tonsil was not assessed in this study, the concentration of enrofloxacin in nasal secretions of pigs is considered nearly equal to plasma concentrations, suggesting that it was not a dose-effect issue (Bimazubute et al., 2009). Additionally, at necropsy, even when cutting the tonsils and collecting swabs from inside the tonsils, pigs treated with enrofloxacin tested negative for *H. parasuis* at 4 and 8 d post-infection, which demonstrates that bacteria was not concentrated deep in the tonsils soon after treatment. Furthermore, antibiotic resistance was not likely since 3 of the 4 *H. parasuis* isolates recovered at necropsy were tested for antimicrobial susceptibility and all 3 isolates were susceptible to enrofloxacin. The fourth isolate would not grow in the test medium.

All pigs remained clinically healthy throughout the study and, as expected, most of the control pigs tested positive in samples from the upper respiratory tract. Furthermore, at necropsy, 5 pigs tested positive in the trachea and 2 pigs tested positive in the lung, which confirms that *H. parasuis* can be detected in the trachea and lung of healthy pigs, as reported elsewhere (Oliveira, 2004). In contrast, swabs

collected at necropsy from pigs treated with antibiotics only tested positive when collected on day 15 of the study.

Pigs in both groups remained serologically negative. The OppA ELISA kit was used to monitor *H. parasuis* systemic infection during the study, since it measures the amount of antibodies to the *H. parasuis* species-specific antigen OppA in pigs systemically infected by all *H. parasuis* serotypes or vaccinated with *H. parasuis*. Healthy (colonized) pigs are not expected to have anti-OppA antibodies. While outside the scope of this study, further investigation is required to determine whether *H. parasuis* strains colonizing the upper respiratory tract are able to trigger an immune response.

Characterization of all *H. parasuis* isolates recovered at necropsy indicated that both groups were colonized with the same *H. parasuis* strain. In addition, the isolates were negative by the *vtaA* group 1 PCR, which indicates that these isolates did not have the *vtaA* virulence factor. This result was expected since *H. parasuis* is a commensal organism of the upper respiratory tract and most of the isolates recovered from the nose are considered non-pathogenic (Oliveira et al., 2003a). One isolate that was *vtaA* negative was obtained from systemic sites (serosas) in a clinically healthy control pig, however, which requires further investigation.

In conclusion, the results of this study indicate that enrofloxacin can reduce the levels of *H. parasuis* in naturally colonized pigs. Reduction of *H. parasuis* in the upper respiratory tract of pigs may help to control the disease during susceptible stages such as the weaning period, possibly delaying the infection to a point when the pigs are

able to develop their own active immunity against this organism. Further research is needed, however, to evaluate the lasting effect of enrofloxacin in the colonization patterns and disease dynamics of *H. parasuis.*

Table 4.1. Daily average colony-forming units (CFUs)/reaction and standard deviation (SD) from pigs in treated and control groups.

	Average CFU/reaction ± (SD)			
	Tonsil		Nasal	
Days after treatment	Control	Treatment	Control	Treatment
0	1.8×10^5 (1.5×10^5)[a]	3.5×10^5 (4.3×10^5)	1.0×10^4 (1.9×10^4)	1.1×10^4 (2.7×10^4)
1	4.0×10^4 (8.0×10^4)	0[c]	6.4×10^3 (8.2×10^3)	0
2	2.0×10^5 (3.8×10^5)	0	1.2×10^4 (1.1×10^4)	0
3	1.5×10^5 (3.3×10^5)	6.8×10^5[b]	6.4×10^4 (1.5×10^5)	0
4	2.3×10^3 (1.7×10^3)	0	3.8×10^4 (2.2×10^4)	0
5	4.6×10^3 (2.8×10^3)	0	4.0×10^4 (3.3×10^4)	0
6	8.0×10^3 (1.3×10^4)	0	7.6×10^3 (1.0×10^4)	0
7	1.8×10^3 (1.8×10^3)	3.9×10^4	1.1×10^4 (1.6×10^4)	0
8	1.8×10^3[b]	0	1.2×10^5 (9.3×10^3)	0
9	7.0×10^3	3.0×10^5	5.1×10^4 (4.2×10^4)	0
10	2.7×10^4 (2.7×10^4)	0	2.0×10^5 (1.2×10^5)	0
11	4.6×10^4 (3.5×10^4)	0	2.3×10^5 (8.8×10^4)	1.8×10^4
12	1.9×10^3 (2.5×10^3)	0	2.9×10^5 (2.7×10^5)	0
13	3.7×10^3 (3.2×10^3)	2.6×10^3 (3.1×10^3)	4.0×10^5 (1.5×10^5)	3.7×10^4
14	2.2×10^3 (1.9×10^3)	2.1×10^3 (1.0×10^3)	2.6×10^5 (3.5×10^4)	9.3×10^4

[a] Average ± standard deviation.

[b] Only one pigs positive in the group.

[c] No positive pigs in the group.

Table 4.2. Number of pigs detected *Haemophilus parasuis* positive by qPCR and bacterial culture at necropsy.

Groups	Control						Treatment					
Day post-treatment	4 (n=4)		8 (n=4)		14 (n=3)		4 (n=4)		8 (n=4)		14 (n=4)	
Nasal	2[a]	(0[b])	4	(0)	3	(2)	0	(0)	0	(0)	1	(1)
Tonsil	2	(0)	3	(0)	2	(0)	0	(0)	0	(0)	3	(0)
Trachea	1	(0)	1	(0)	3	(0)	0	(0)	0	(0)	1	(0)
Lung	1	(0)	0	(0)	1	(0)	0	(0)	0	(0)	0	(0)
Serosae	0	(0)	0	(0)	0	(1)	0	(0)	0	(0)	0	(0)

[a] qPCR

[b] Bacterial culture

Figure 4.1. Percentage of positive pigs by nasal swabs qPCR from control (n=11) and treatment (n=12) groups by day. [a] Differences ($p<0.003$) between treatment and control.

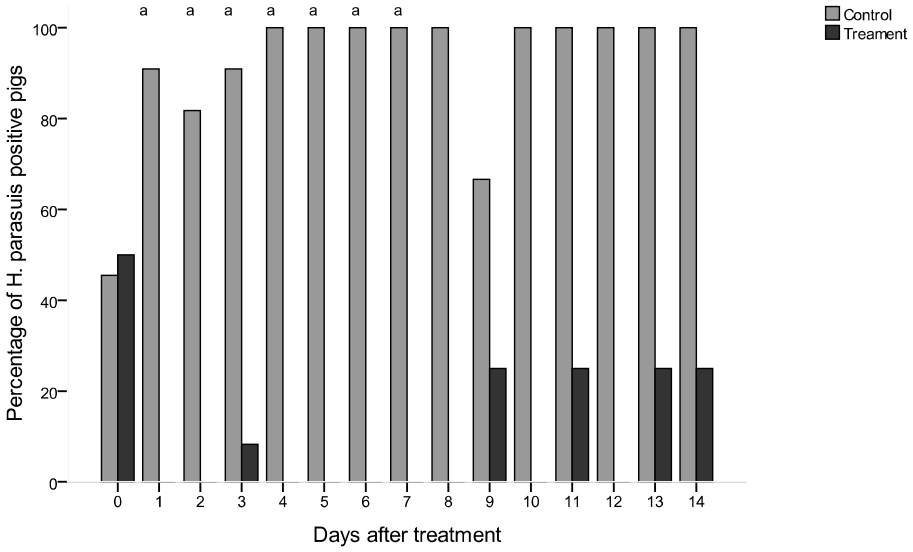
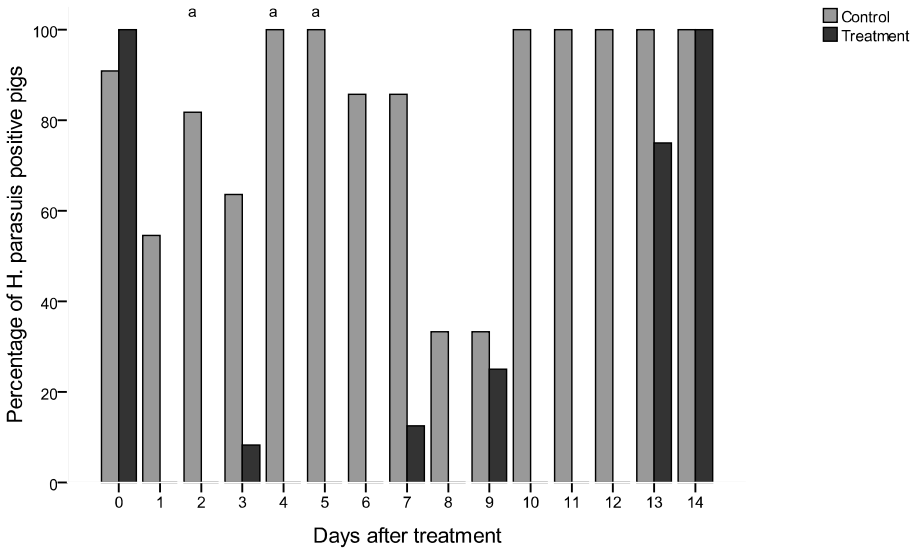


Figure 4.2. Percentage of positive pigs by tonsil swabs qPCR from control (n=11) and treatment (n=12) groups by day. [a] Differences ($p<0.003$) between treatment and control.



CHAPTER 5

EFFECT OF ENROFLOXACIN ON *HAEMOPHILUS PARASUIS* INFECTION, DISEASE AND IMMUNE RESPONSE

Macedo N, Cheeran M, Rovira A, Torremorell M. Effect of enrofloxacin on *Haemophilus parasuis* infection, disease and immune response. [In preparation]

Abstract

Haemophilus parasuis is a pathogen that colonizes the upper respiratory tract (URT) of pigs and, through mechanisms not well understood, invades the bloodstream and causes polyserositis. This syndrome is known as Glasser's disease. Antimicrobial treatment is widely used to treat Glasser's disease, but the effects of early elimination of *H. parasuis* before immunity is activated are not known. We characterized the antibody and IFN-γ responses to *H. parasuis* in pigs treated with enrofloxacin before or after low dose inoculation with a pathogenic *H. parasuis* strain to better understand the effect of enrofloxacin on immune response to *H. parasuis*. Pigs that were inoculated only (EXP group) and pigs that were treated with enrofloxacin and then inoculated (ABT/EXP group) developed signs of disease starting at 4 days post inoculation (DPI). Pigs from these groups also presented a significant increase on levels of serum IgG. This seroconversion was associated with protection against challenge. In contrast, pigs treated after inoculation (EXP/ABT group) did not have signs of disease or seroconverted after inoculation. EXP/ABT pigs as well as naïve control pigs [enrofloxacin only (ABT) and challenge only (CHA)] were susceptible to challenge. Variable levels of serum IgA antibodies, IgG and IgA in bronchioalveolar fluid (BALF) and IFN-γ responses were observed after *H. parasuis* inoculation in the different groups, but the values were not associated with protection. After low dose inoculation, the virulent *H. parasuis* strain was isolated from most of inoculated pigs. However, enrofloxacin given after *H. parasuis* inoculation eliminated *H. parasuis* from the noses and larynges of treated pigs. In summary, enrofloxacin treatment 3 days before *H. parasuis* inoculation did not interfere with *H. parasuis* infection and subsequent seroconversion and protection against challenge. However, pigs treated

with enrofloxacin after *H. parasuis* inoculation did not seroconvert and were susceptible to challenge.

Introduction

Haemophilus parasuis is a Gram-negative bacterium that causes Glasser's disease in pigs. The disease is characterized by fibrino-purulent polyserositis, arthritis and meningitis leading to high mortality and morbidity, which results in significant economic losses to pig producers. Pathogenic and non-pathogenic *H. parasuis* strains can be isolated from the upper respiratory tract of healthy pigs (chapters 3 and 4). Stress conditions such as weaning and transport have been suggested as risk factors for *H. parasuis* systemic dissemination (Aragon et al., 2012). However, the mechanisms involved in the systemic invasion of *H. parasuis* are largely unknown.

Vaccines against *H. parasuis* are available and can be used for prevention. However, control of Glasser's disease through vaccination can be difficult, especially because there are many strains and subtypes, and cross-protection among isolates is limited. In contrast, field studies demonstrated that inoculating piglets with a low dose of a live pathogenic *H. parasuis* strain reduced nursery mortality due to Glasser's disease (Oliveira et al., 2001; Oliveira et al., 2004). The mechanism behind such protection was not determined, but it was hypothesized that such exposure results in *H. parasuis* colonization, which may elicit a protective immune response without causing disease (Pijoan et al., 1997).

Antimicrobials have been widely used in the swine industry to control bacterial respiratory diseases (Cromwell, 2002). About half of farms in the US with nursery-

age pigs use injectable antimicrobials to treat respiratory diseases. The most common action for pigs with clinical respiratory disease is to administer antimicrobials to all pigs in the entire room, according to data from the National Animal Health Monitoring System Swine 2006 study (Anonymous, 2006). Antimicrobials exert a direct deleterious effect over bacterial infections by decreasing the bacterial load and permitting the host to activate immune defenses and eliminate the pathogen without excessive inflammation (Cromwell, 2002). Specifically for *H. parasuis,* antimicrobials are extremely useful in the control and treatment of Glasser's disease (Aragon et al., 2012). On the other hand, there are few reports indicating that antimicrobials can have unintended consequences by preventing the development of immunity, as described below. Antimicrobial treatment in the early stages of disease prevented the development of protective immunity against reinfection with *Listeria sp.* in mice*, Chlamydia trachomatis* in mice, *Actinobacillus pleuropneumoniae* in pigs and *Salmonella typhimuium* in mice (North et al., 1981; Su et al., 1999; Sjolund et al., 2009; Griffin et al., 2009). In contrast, a recent study demonstrated that early antibiotic treatment with enrofloxacin against *S. typhimurium* infection in mice primed a specific antibody response, which protected against secondary challenge (Johanns et al, 2011). Antimicrobial treatment can also affect *H. parasuis* colonization (chapter 4, Vilalta et al., 2012). However, the effect of antimicrobials on the development of an effective immune response against *H. parasuis* requires further investigation.

To better study the relationship between *H. parasuis* infection, immunity, protection and use of antibiotics, we used an animal model where conventional pigs were inoculated with a low dose of a pathogenic *H. parasuis* strain at weaning and were

treated with enrofloxacin, followed by homologous challenge. To our knowledge, there is no information available on how antimicrobials affect protection against *H. parasuis* in pigs experimentally colonized with a pathogenic *H. parasuis* strain. Our hypothesis is that enrofloxacin treatment will affect *H. parasuis* infection and subsequent development of a protective immune response against *H. parasuis*.

Material and Methods

Experimental design

Sixty 3-week-old conventional pigs from seven different litters were selected from a specific pathogen free herd without history of Glasser's disease. Pigs were individually identified, assigned to 6 groups of 10 pigs each, and equally distributed as far as possible in terms of sex, weight and litter of origin. Each group was kept in a separate isolation room and pigs were cared according to University of Minnesota approved Institutional Animal Care and Use Committee (IACUC) protocols.

A summary of the experimental procedures for each group is shown in Figure 5.1 and Table 5.1. Three groups of pigs (EXP, EXP/ABT, ABT/EXP) were inoculated with a low dose of pathogenic *H. parasuis* (exposure) on day 0. Group ABT/EXP was also treated with enrofloxacin before inoculation on day -3 of the study. Group EXP/ABT was treated with enrofloxacin 3 days after inoculation. Group ABT was also treated with enrofloxacin on day 3 but was not exposed to low dose of pathogenic *H. parasuis,* and served as a control for the effect of the antibiotic treatment alone. Pigs in group CHA were only inoculated with a high dose of *H. parasuis* (challenge) on day 21 and served as positive controls. Groups EXP, EXP/ABT, ABT/EXP and ABT

were also challenged on day 21. Pigs in the negative control group (NEG) were untreated, non-exposed and non-challenged.

At the termination of the study, at 25 or 35 DPI, pigs were euthanized and necropsied for pathological evaluation. Pigs showing severe clinical signs of disease including lethargy, respiratory distress, lameness or lateral recumbency were euthanized and necropsied as soon as severe clinical signs were identified.

Haemophilus parasuis inoculation

H. parasuis Nagasaki strain was selected for inoculation of piglets. This is a highly pathogenic reference strain extensively described in the literature (Cerda-Cuellar and Aragon, 2008; Aragon et al., 2009; Olvera et al., 2009; 2012; Frandoloso et al., 2012; 2013). The Nagasaki strain had an in vivo passage using chicken eggs to increase its virulence (Oliveira and Pijoan (2002). Bacterial growth was harvested from chocolate agar plates, quantified, aliquoted and frozen at -80°C. Prior to inoculation, cultures were diluted to produce an inoculum containing 10^6 (exposure at day 0) or 10^8 (challenge at day 21) colony forming units (CFU)/ml of Nagasaki strain.

Inoculation was performed as described in chapter 4. Pigs inoculated at the beginning of the study received 1.0 mL of inoculum containing 10^6 CFU/ml of Nagasaki strain intranasally (0.5 mL in each nostril) at day 0. Non-inoculated pigs received 1.0 mL of sterile saline intranasally. Before inoculation, pigs were sedated by an intramuscular injection in the cervical muscles of a dissociative anesthetic at recommended dose of 6.6 mg/kg (0.06 ml/kg) (Telazol®, Fort Dodge Animal Health, Fort Dodge, IA). Once sedation was achieved (1-2 minutes), pigs were held upright and the inoculum was

instilled slowly into each nostril using a 1 mL syringe. Inoculation with high challenge dose was similarly performed on day 21 of the study, using an inoculum containing 10^8 CFU/mL of Nagasaki strain.

Antimicrobial treatment

Pigs treated with antimicrobial received a single dose of injectable enrofloxacin (7.5 mg/kg of body weight, Baytril, Bayer Animal Health, Shawnee, Kansas, USA), subcutaneously, either 3 days prior (ABT/EXP) or post (EXP/ABT) low dose inoculation (Table 5.1). Pigs not treated received 1 mL of saline solution.

Pigs that had clinical signs of Glasser's disease, including body temperature above 40° C, lethargy, respiratory distress, lameness or lateral recumbency were treated therapeutically with enrofloxacin following manufacturer specifications as described above.

Sample collection

Nasal swabs for bacterial isolation were collected at -3, 2, 7 and 17 DPI from all pigs in all groups and at 3 and 5 DPI from pigs in groups EXP, EXP/ABT and ABT/EXP. Laryngeal swabs were collected from groups EXP, EXP/ABT and ABT/EXP only at 2, 3, 6, 7 and 18 DPI. Swabs of nasal cavity, trachea, brain, pleura, pericardium, peritoneum and joints were collected at necropsy for *H. parasuis* isolation. *H. parasuis* isolation was also attempted from lung and liver tissues. Blood samples were collected at 2 and 23 DPI from exposed pigs to monitor for bacteremia through bacterial isolation and PCR (Oliveira et al., 2001b). Serum samples were collected at -3, 17, 25 and 35 DPI for serological testing. Blood samples were also collected in EDTA tubes (BD Vacutainer™ Glass Blood Collection tubes with K_3 EDTA;

Franklin Lakes, NJ, USA) on days 0, 7, 15, 25 and 35 to evaluate the number of peripheral blood mononuclear cells (PBMCs) producing IFN-γ upon *H. parasuis* stimulation by Enzyme-Linked ImmunoSpot (ELISPOT).

Clinico-pathological evaluation

Body temperature and clinical signs compatible with Glässer's disease (cough, lethargy, abdominal breath, lameness and lateral recumbency) were recorded daily after *H. parasuis* inoculation on day 0. Pigs showing signs of advanced Glasser's disease were euthanized by a pentobarbital overdose (100 mg/kg, Fatal-Plus Solution®, Vortech Pharmaceuticals, Dearborn, MI, USA). At the termination of the study, at 25 or 35 DPI, pigs were euthanized and necropsied for evaluation of gross and microscopic lesions. Lesions of pulmonary consolidation, presence of fibrin in abdomen, thorax and joints were recorded. Mortality associated to *H. parasuis* was confirmed by bacterial isolation from the lesions followed by genotyping by enterobacterial repetitive intergenic consensus (ERIC-PCR).

H. parasuis isolation and characterization

For isolation of *H. parasuis*, samples were plated onto sheep blood agar streaked with a nurse *Staphylococcus aureus* strain, and incubated at 37°C in a 5% carbon dioxide (CO2) atmosphere for 24 to 48 hours. Suspect colonies showing satellitism to the *S. aureus* nurse streak were isolated onto a new blood agar plate, incubated in similar conditions for 24 additional hours, and characterized through biochemical testing (Oliveira et al., 2003).

H. parasuis isolates were genotyped by ERIC-PCR (Rafiee et al., 2000) in order to distinguish between the Nagasaki strain and naturally colonizing commensal *H. parasuis*. Additionally, a multiplex PCR based on detection of the gene encoding virulence factor vtaA was used to differentiate non-pathogenic from potentially pathogenic *H. parasuis* isolates (Olvera et al, 2011).

Detection of H. parasuis-specific IgG and IgA by ELISA

To study antibody responses to *H. parasuis*, *H. parasuis* recombinant oligopeptide permease A (rOppA) protein was obtained as described previously (chapter 2) from GenScript Corp., Piscataway, New Jersey. The OppA protein is highly immunogenic, species-specific, conserved among *H. parasuis* strains, and is recognized by serum from pigs systemically infected with a wide variety of *H. parasuis* strains and serotypes (chapter 2).

An in house ELISA to detect antibodies against the OppA was developed as described in chapter 2. Briefly, high binding plates were coated with 100 nanograms/well of recombinant OppA (Genscript) and blocked with 5% skim milk. Sera were diluted 1:50 for IgG and IgA detection. Bronquioalveolar lavage fluid (BALF) was diluted 1:8. Rabbit anti-porcine IgG HRP (Sigma-Aldrich, St. Louis, MO, USA) was diluted 1:50000 and goat anti-porcine IgA HRP (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1:3200. Antibodies were detected with peroxidase substrate (KPL, Gaithersburg, MD, USA). The plates were read at 450 nm using a microplate reader (ThermoMax Molecular Devices, Sunnyvale, CA).

Isolation, freezing and thawing of porcine PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation of blood diluted 1:2 with sterile phosphate buffered saline (PBS) (Cell Signaling Technology, Danvers, MA, USA) on lymphocyte separation medium (Mediatech, Manassas, VA, USA). PBMCs were washed with PBS, and freed of red blood cells by ammonium-chloride-potassium (ACK) lysis buffer (Lonza, Alpharetta, GA, USA). Cells were resuspended in RPMI complete medium containing 1mM sodium pyruvate (Life Technologies, Grand Island, NY, USA), penicillin (100 U) and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA). Cell viability was confirmed by trypan blue exclusion, and cells were counted using the Cellometer® Auto Counter T4 (Nexcelom Bioscience LLC, Lawrence, MA, USA). Cells were resuspended to 10^7 cell/mL with freezing solution containing 70% FBS, 20% cRPMI, 10% Dimethyl Sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and kept on liquid nitrogen until use. Cells were thawed in 37°C water bath and washed with RPMI containing 5% FBS and 50 U/mL of benzonase nuclease (Sigma-Aldrich, St. Louis, MO, USA). Cells were resuspended with 1 mL of RPMI containing 10% FBS, 1mM sodium pyruvate (Life Technologies, Grand Island, NY, USA), penicillin (100 U) and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and added to plates at specified concentration.

ELISPOT assay for IFN-γ response

As a measure of cell-mediated immune response to *H. parasuis*, IFN-γ production responses were assessed by in vitro stimulation of PBMCs with *H. parasuis* outer membrane proteins (OMPs) and mitogens for 24 hours, followed by measuring antigen-specific, actively secreting IFN-γ producing cells.

The method used for detergent-insoluble OMP extraction from *H. parasuis* has been described by Ruiz et al. (2001). Briefly, an overnight culture of *H. parasuis* Nagasaki strain in PPLO medium supplemented with 5% horse serum and 40 µg of NAD/mL was centrifuged for 10 minutes at 5,000 x g. The pellet was resuspended in 10 mM HEPES buffer (pH 7.4), and the suspension was subjected to sonication for 2 minutes at 22 µm amplitude (sonicator) in ice. Sonication was repeated until solution changed color from cloudy, turbid to translucent. Cellular debris was removed by centrifugation at 20,000 x g for 3 minutes at 4°C. The supernatant was then removed and centrifuged at 20,000 x g for 40 minutes at 4°C. The pellet containing the cell membrane material was resuspended in HEPES buffer and sodium lauryl sarcosinate (Sigma-Aldrich, St. Louis, MO, USA) (2% in HEPES buffer) for 30 minutes with intermittent mixing. After that, each preparation was centrifuged at 20,000 x g for 40 minutes at 4°C. The membrane pellet was then resuspended in HEPES buffer to a concentration of 100 µg/mL and stored at -80°C.

Microtiter PVDF plates (Millipore MultiScreen™, EMD Millipore Corporation, Billerica, MA, USA) were coated with 100 µL/well of 1:60 dilution of anti-porcine IFN-γ capture antibody (R&D Systems, Inc., Minneapolis, MN, USA) in PBS overnight at 4°C and blocked by adding 200 µL/well of 1% bovine serum albumin (BSA) in PBS. Cells were diluted in RPMI 1640 to a density of 500,000 cells per well in a volume of 50 µL/well. *H. parasuis* OMPs recall antigen (25 µg/well), 10 µg/mL of Concanavalin A (ConA) (positive control) (Sigma-Aldrich, St. Louis, MO, USA) or 25 µg/well of Hemocyanin from *Megathura crenulata* (keyhole limpet) KLH (Sigma-Aldrich, St. Louis, MO, USA) (negative control) was added to each well. Plates were incubated for 24 hours at 36°C in a CO_2 incubator. Medium was removed,

and plates were washed six times with phosphate buffered saline with Tween-20 (PBST) (Cell Signaling Technology, Danvers, MA, USA). One hundred mL/well of a 1:100 dilution of anti-porcine IFN-γ detection antibody (R&D Systems, Inc., Minneapolis, MN, USA) were added in PBS with 1% BSA and incubated for 2 hours at room temperature (RT). Plates were washed again six times with PBST, and 100 μL of 1:100 dilution of streptavidin-alkaline phosphatase in 1% BSA was added for 2 hours at RT. After 4 washes, 100 μL/well of BCIP/NBT chromogen (R&D Systems, Inc., Minneapolis, MN, USA) was added and incubated in the dark for 30 minutes at RT, then rinsed with water and let dry at RT. Spots were quantified automatically by using a specialized automated ELISPOT reader (ImmunoSpot® 5.1 software, C.T.L. Analyzers, LLC, Cleveland, OH, USA). Data are presented as the mean numbers of *H. parasuis*-specific IFN-γ producing cells per 10^6 PBMCs from duplicate wells of each sample minus the number of cells on wells simulated by KLH.

Statistical analysis

The presence of clinical signs and lesions at necropsy were analyzed for significant differences between groups using Fisher's exact test ($p<0.05$). Serology and ELISPOT data were statistically analyzed for differences between groups using Kruskal-Wallis test followed by Mann-Whitney pairwise comparison ($p<0.05$). Serology and ELISPOT data were also compared within groups by repeated measures Friedman's ANOVA test, followed with multiple pairwise comparisons performed using Wilcoxon signed rank test ($p<05$). Survival analysis was used to compare the time of Nagasaki isolation from URT and the onset of clinical signs after *H. parasuis* inoculation. Differences between groups were analyzed by Cox Regression ($P<0.05$).

Results

Clinico-pathological outcomes to H. parasuis inoculation

After low dose inoculation, clinical signs were observed in 6/10 pigs from the EXP group and 5/10 pigs from the ABT/EXP group. These proportions of pigs with clinical signs were not statistically different from each other (Table 5.2), but were different from the EXP/ABT and the non-exposed groups (ABT, CHA and NEG) ($p<0.05$). Pigs in the other four groups did not show clinical signs.

Four pigs from the EXP group had fever above 40°C at 4 DPI. Two of the affected pigs were euthanized and the other two were treated therapeutically with enrofloxacin as previously described. At 8 DPI, two other pigs from the EXP group presented fever and were immediately treated therapeutically (Table 5.2). The body temperatures of the therapeutically treated pigs returned to normal after 24 hours and they were kept in the study.

Four pigs from the ABT/EXP group had temperature above 40°C after low dose inoculation, starting at 7 DPI. These pigs were similarly treated with enrofloxacin and their body temperature normalized 24 hours post treatment. However, one of the therapeutically treated pigs was found dead at 16 DPI. In addition, another pig from the same group was found in distress (lethargy and hypothermia) and was therefore euthanized at 7 DPI (Table 5.2). Although onset of clinical signs was observed sooner in group EXP (4- 8 days) compared to group ABT/EXP (7-8 days) (Table 5.2), no significant differences in the onset of clinical signs were found by Cox regression.

Necropsy of the two pigs from group EXP euthanized at 4 DPI revealed moderate arthritis in the hock joints of both posterior legs in pigs. The pig from group ABT/EXP euthanized on day 7 had mild peritonitis while the pig found dead on day 16 did not have any lesions at necropsy. Nevertheless, *H. parasuis* was isolated from systemic sites from all necropsied pigs and confirmed as Nagasaki strain according to the ERIC-PCR pattern.

Results on clinical outcomes after high dose challenge inoculation can be seen in Table 5.3. Pigs from the NEG, EXP and ABT/EXP groups did not show any signs of disease. In contrast, 4 out of 10 pigs in the EXP/ABT and CHA groups and 8 out 10 pigs in the ABT group had clinical signs of disease after challenge, including fever, lethargy, coughing, respiratory distress, lameness and lateral recumbency. The proportion of affected pigs in groups EXP/ABT, ABT and CHA was statistically higher when compared to non-affected pigs (EXP, ABT/EXP and NEG groups) ($p<0.05$) (Table 5.3). Five pigs (4 from ATB and 1 from CHA groups) were euthanized before the first endpoint of the study (day 25 DPI) due to severe clinical signs (Table 5.3). At first necropsy day (25 DPI or earlier), 14 pigs (6 ABT, 4 CHA and 4 EXP/ABT) had lesions characteristic of Glasser's disease, including fibrinopurulent pericarditis, pleuritis, peritonitis, arthritis, and pneumonia. Nagasaki strain was recovered from all 14 pigs from trachea, lung, liver or serosal sites (Table 5.4 and Table 5.5). In addition, Nagasaki strain was recovered from the nose of only four affected pigs (1 EXP/ABT, 2 ABT and 2 CHA) (Table 5.4 and Table 5.5).

Nasal, tracheal and serosal swabs collected at necropsy from the pigs from groups that did not show disease (NEG, EXP, ABT/EXP) tested negative for the Nagasaki strain.

Pigs that were not euthanized on 25 DPI remained healthy until day 35 DPI, and no lesion was observed during the second necropsy on 35 DPI. The only Nagasaki isolate recovered on 35 DPI was from the nose of two pigs from CHA group.

***H. parasuis* isolation from the URT**

H. parasuis was isolated from nasal and laryngeal swabs of 54/60 pigs (90%) yielding 142 isolates before challenge. Of those, 34 isolates were identified by ERIC-PCR as the pathogenic *H. parasuis* Nagasaki strain, which had been inoculated into the pigs. Overall, there were 28 Nagasaki *H. parasuis* isolates recovered from nasal swabs and 6 from laryngeal swabs. The other 108 isolates were identified as a different strain that all pigs carried in the nasal cavity, and is referred to as "farm strain" hereafter. Nagasaki strain was differentiated from the farm strain by its unique ERIC-PCR fingerprint (chapter 2). Additionally, the Nagasaki strain was positive for the virulent vtaA gene (chapter 2), while the farm strain was not.

The Nagasaki strain was cultured from nasal or laryngeal swabs from 8/10 pigs in group EXP, 9/10 pigs in group ABT/EXP and 5/10 pigs in group EXP/ABT before challenge. These differences in the proportion of pigs with positive URT cultures were not significantly different (Table 5.6). The time of first isolation ranged from 2 to 7 DPI for pigs in group EXP, 2 to 7 in group ABT/EXP and 2 to 3 in group EXP/ABT. Although there was a delay in pigs yielding Nagasaki isolates in ABT/EXP group compared to EXP and EXP/ABT groups (Table 5.6), there were no significant differences in time to Nagasaki isolation by Cox regression ($p>0.05$).

The farm strain was isolated from 48/60 (80%) pigs, resulting in 108 isolates recovered before challenge (Table 5.7). The rate of isolation of the *H. parasuis* farm strain at the beginning of the study (-3 DPI) ranged between 40% and 80% in the different experimental groups (Table 5.7). These differences were not significantly different. The *H. parasuis* farm strain continued to be isolated throughout the study from the URT of pigs that were not treated with antibiotics (EXP, NEG and CHA). In contrast, *H. parasuis* farm strain was not isolated from pigs in groups EXP/ABT, ABT/EXP and ABT after enrofloxacin administration.

Serological immune response

To test whether inoculation with a low dose of pathogenic *H. parasuis* induced IgG and IgA antibodies against *H. parasuis* immunogen OppA, serum samples were analyzed by an in house *H. parasuis* ELISA. After low dose inoculation, serum IgG antibodies increased in pigs from groups EXP (average OD of 1.13) and ABT/EXP (average OD of 0.99) on day 17 compared with day -3 (average OD of 0.17 and 0.18, respectively) ($p<0.05$) (Figure 5.2). Additionally, levels of serum IgG antibodies were higher for groups EXP and EXP/ATB on day 17, when compared to group EXP/ABT and non-inoculated groups ($p<0.05$). No significant increases in serum IgG antibodies were observed among the pigs from EXP/ABT group and control groups before challenge.

Levels of serum IgA antibodies were significantly higher on day 17 for all groups, compared to day -3 DPI ($p<0.05$). A significant difference on IgA levels in serum was not observed between groups on day 0 or day 17 ($p>0.05$) (Figure 5.3).

After challenge (25 and 35 DPI), levels of serum IgG remained high for groups EXP and ABT/EXP (Figure 5.2). The levels of serum IgG antibodies significantly increased in pigs from groups EXP/ABT, ABT and CHA 14 days after challenge (35 DPI) when compared to 25 DPI ($p<0.05$). Levels of serum IgG remained low in the NEG group throughout the study and significantly lower than the other groups on 35 DPI (Figure 5.2). Levels of serum IgA were statistically higher for all groups compared to NEG group on 35 DPI ($p<0.05$) (Figure 5.3).

There were no differences between groups on IgG or IGA levels in BALF on day 25 DPI. Levels of IgG in BALF were statistically higher in pigs from all challenged groups on day 35 when compared to NEG group ($p<0.05$) (Figure 5.4). However, no significant differences were observed among groups on the levels of IgA antibodies in BALF at 35 DPI (Figure 5.5).

IFN-γ immune response

The *H. parasuis*-specific cell-mediated immune response was assessed using an enzyme-linked immunosorbent spot assay (ELISPOT) designed to detect IFN-γ producing PBMCs. No differences on the number of IFN-γ producing cells were observed between groups on day -3. Pigs in EXP and ABT/EXP groups showed a statistically higher number of *H. parasuis*-specific IFN-γ producing cells at 7 and 15 DPI, respectively, compared to day -3 ($p<0.05$) (Figures 5.6). In addition, on day 7, EXP group had significantly higher IFN-γ response compared to NEG group, while ABT/EXP group had a higher IFN-γ on day 15 DPI, when compared to other groups ($P<0.05$). A significant IFN- γ response was not observed in EXP/ABT and non-exposed groups (ABT, CHA and NEG) after low dose inoculation.

After challenge, on 25 DPI, levels of *H. parasuis*-specific IFN-γ producing cells were statistically higher for all groups when compared with NEG group ($p<0.05$), (Figure 5.6). By 35 DPI, no differences on IFN- γ responses were observed between groups.

Discussion

There is limited information on how antimicrobials modify the susceptibility of pigs to *H. parasuis* infection. In this study, we evaluated the use of enrofloxacin in relation to the timing of *H. parasuis* infection and the predisposition to Glasser's disease. Our results indicated that enrofloxacin given 3 days after pigs were exposed controlled infection before pigs were able to activate a protective immune response. In contrast, enrofloxacin given 3 days before inoculation did not interfere with the development of an immune response, resulting in pigs being protected against challenge.

Inoculation of pigs with a low dose of pathogenic *H. parasuis* resulted in most pigs being colonized by the pathogenic strain and having an increase on levels of IgG antibodies in serum. In addition, all pigs from EXP group were protected against challenge. Protection against Glasser's disease after exposure to low dose of pathogenic *H. parasuis* is, to some extent, discordant in the literature. A different study reported that exposure to *H. parasuis* pathogenic strain at a young age resulted in protection from clinical disease in a farm setting (Oliveira et al., 2004). In contrast, when pigs were inoculated with a low dose of pathogenic *H. parasuis* in another study, only partial protection (3 out of 5 pigs) was reported after challenge with a high dose of the same strain (Martin de la Fuente et al., 2009a). Whether a subclinical or systemic infection by *H. parasuis* is needed to activate a protective immune response

or whether only colonization by a pathogenic *H. parasuis* strain would be enough to prime the immune system still needs further investigation.

The protection observed by a low dose inoculation with *H. parasuis* was not affected by enrofloxacin given 3 days before Nagasaki inoculation. Similar to EXP group, most pigs from ABT/EXP group were colonized with the pathogenic strain and had increased levels of serum IgG antibodies. Enrofloxacin is a fluoroquinolone that acts by inhibiting DNA gyrase, an enzyme responsible for bacterial replication, which leads to cell death. Enrofloxacin concentration peaks in plasma and nasal secretions of pigs between 1 and 2 hours after administration with terminal half-life of about 9 and 12 hours in plasma and nasal secretions respectively (Bimazubute et al., 2009). Even though the duration of effective concentration of enrofloxacin in plasma and nasal secretions of swine is not known, data from this study suggests that the concentration of enrofloxacin 3 days after administration must have been low enough to allow the establishment of *H. parasuis* infection in pigs.

On the contrary, enrofloxacin given 3 days after inoculation with the pathogenic strain interfered with protection against challenge. Neither the farm nor the Nagasaki *H. parasuis* strains were isolated from the URT of EXP/ABT group after enrofloxacin treatment. In addition, these pigs did not develop disease after colonization and an increase of IgG antibodies was not observed at 17 DPI. Finally, 40% of those pigs were susceptible to challenge on 21 DPI. Based on lack of *H. parasuis* isolation after enrofloxacin treatment, it appears that the antibiotic quickly prevented the immune response by inactivating and removing *H. parasuis*. In fact, besides its broad distribution on plasma and tissues, enrofloxacin also actively accumulates in

phagocytes and quickly eliminates susceptible bacteria by enhancing intraphagocytic killing (Schoevers et al., 1999). Alternatively, the enrofloxacin treatment may have had a direct immunosuppressant effect on the immune system of injected pigs. Fluoroquinolones are known to have modulating effects on the immune response, by decreasing the synthesis of pro-inflammatory cytokines, such as IL-1 and TNF (Khan et al., 2000), which could have contributed to the absence of immune response in group EXP/ABT. More research is needed, however, to investigate whether such immunomodulatory effects would affect the immune response to *H. parasuis*.

Groups EXP and ABT/EXP had significantly higher levels of IgG antibodies in serum at 17 DPI and were protected against challenge at 21 DPI. The groups that were seronegative at 17 DPI (EXP/ABT, ABT and CHA), showed an increase of IgG antibodies in serum at 31 DPI. In addition, all challenged groups had higher levels of IgG antibodies in serum and BALF than non-challenged NEG group ($p<0.05$). Therefore, pigs systemically infected by a pathogenic *H. parasuis* strain develop serum IgG antibodies, which are the main immune correlate of protection from Glasser's disease in this study. This finding is in agreement with several previous reports (Solano-Aguilar et al., 1999; Martin de la Fuente et al., 2009; Nedbalcova et al., 2011).

On the other hand, an increase on serum IgA was observed for all groups at 17 DPI compared to day -3. The cause of this increase in IgA remains unknown. No differences were observed between groups until 35 DPI, when all challenged groups had higher levels of serum IgA compared to the NEG group. In BALF, no differences on IgA levels were observed between groups on 25 and 35 DPI. In other studies, pigs

vaccinated with a killed *H. parasuis* vaccine were protected against challenge, and serum IgG immunity developed without a robust serum IgA antibody response (Olvera et al., 2013; Martinez-Martinez et al., 2013). Apparently, serum IgA response is activated after *H. parasuis* systemic infection or vaccination, but does not seem to play a significant role on protection against disease.

Moreover, an average increase of IFN-γ producing cells was observed on EXP group at 7 DPI when compared to NEG group, and on ABT/EXP group at 15 DPI when compared to all the other groups (Figure 5.6). The higher levels of IFN-γ producing-cells are apparently associated with onset of clinical signs in those two groups. In a different study, an increase of IFN-γ concentrations was also described in the sera of piglets after vaccination (Hu et al., 2013). In the present study, no increase of IFN-γ producing cells was observed in EXP/ABT after low dose inoculation, probably because the infection was eliminated before an immune response could be developed. Furthermore, quinolones antimicrobials have been shown to decrease IFN-γ expression in human T lymphocytes under in vitro conditions (William et al., 2005), with potential implications for immune response and recovery after severe infection, but the mechanisms of such effects need further investigation in vivo. After challenge, all groups had a significantly higher IFN-γ response at 25 DPI when compared to NEG group. The number of IFN-γ producing cells was not different from background on day 35, apparently because IFN-γ response seems to be transient in blood and it is dissipated after peak of infection from days 22 to 25 (1 to 4 days after challenge).

In summary, in this study we demonstrated that antibiotic treatment can alter the development of a protective immune response for *H. parasuis* and that this effect is

dependent on the timing of antibiotic administration relative to infection. In addition, it is possible to speculate on the relative role of the different immune responses in the observed protection against *H. parasuis* challenge: serum IgA and IFN-γ producing-cells did not appear to play a significant role. In contrast, serum IgG levels were associated with protection. Protection against re-infection is highly desirable in commercial herds, where repeated exposures to *H. parasuis* are common. Controlling disease with potent antimicrobials such as enrofloxacin before pigs had time to develop a protective immune response might result in clinical disease if pigs become reinfected. On the other hand, treating pigs when clinical signs of disease are evident does not seem to interfere with protection against re-infection because the immune response may already have been activated. Overall more work on understanding the immune response against *H. parasuis* infection is needed to understand the protective immune mechanisms against *H. parasuis* infection. In addition, studies including different antimicrobials would provide valuable information to better assess appropriate timing and judicious use of antimicrobial treatment in pigs.

Table 5.1. Experimental design summary.

		Days of the study				
Groups	Number of pigs[a]	-3	0	3	21	25/35
EXP	10		Exposure[c]		Challenge[d]	Necropsy
ABT/EXP	10	Enrofloxacin[b]	Exposure		Challenge	Necropsy
EXP/ABT	10		Exposure	Enrofloxacin	Challenge	Necropsy
ABT	10			Enrofloxacin	Challenge	Necropsy
CHA	10				Challenge	Necropsy
NEG	10					Necropsy

[a] 3-week-old, high-health pigs

[b] One dose of 7.5 mg/kg injectable enrofloxacin (Baytril® 100)

[c] Inoculation with *H. parasuis* Nagasaki strain at a low dose of 10^6 CFU/ml

[d] Inoculation with *H. parasuis* Nagasaki strain at a high dose of 10^8 CFU/ml

Table 5.2. Clinical outcome after low dose *Haemophilus parasuis* Nagasaki strain inoculation.

Group	Number of inoculated pigs	Number of clinically afftected pigs[1]	Days from inoculation to clinical signs
EXP	10	6[a]	4,4,4,4,8,8
ABT/EXP	10	5 [a]	7,7,8,8,8
EXP/ABT	10	0 [b]	-
ABT	0	0 [b]	-
CHA	0	0 [b]	-
NEG	0	0 [b]	-

[a] Clinical signs included fever > 40°C, prostration, swollen joints, respiratory distress, and lateral recumbency.

Table 5.3. Proportion of pigs positive for *Haemophilus parasuis* Nagasaki strain isolation after challenge (23 to 35 DPI).

Group	Nose	Trachea	Lung	Serosa
EXP	0/8	0/8[a]	0/8	0/8 [a]
ABT/EXP	0/8	0/8[a]	0/8	0/8 [a]
EXP/ABT	1/10	4/10[b]	0/10	2/10 [a]
ABT	2/10	5/10[b]	5/10	5/10 [b]
CHA	2/10	6/10[b]	3/10	2/10 [a]
NEG	0/10	0/10 [a]	0/10	0/10 [a]

Different letters indicate significant differences between groups ($p<0.05$).

Table 5.4. Clinical outcome after challenge by inoculation with a high dose of *Haemophilus parasuis* Nagasaki strain.

Group	Number of clinically afftected pigs[1]	Days from challenge to clinical signs	Presence of lesions at necropsy
EXP	0/8[a]	-	0/8 [a]
ABT/EXP	0/8[a]	-	0/8 [a]
EXP/ABT	4/10[a]	3,3,3,3	4/10 [b]
ABT	8/10[b]	2,2,3,3,3,3,3,4	6/10 [b]
CHA	4/10[a]	2,2,3,3,	4/10 [b]
NEG	0/10[a]	-	0/10 [a]

[1]Clinical signs included fever > 40°C, prostration, swollen joints, respiratory distress, and lateral recumbency.

Different letters indicate significant differences between groups ($p<0.05$).

Table 5.5. Isolation of *Haemophilus parasuis* Nagasaki strain from pigs with lesions at necropsy (23 to 35 DPI).*

Groups	Pig no.	Days post-challenge	Necropsy specimens†							
			Ns	Tr	Pl	Lu	Pc	Lv	Pt	Jt
EXP/ABT	57	4	-	+	+	-	+	-	-	-
	23	4	+	+	-	-	-	-	-	-
	56	4	-	+	+	-	-	-	-	-
	9	4	-	+	-	-	-	-	-	-
ABT	19	2	+	+	+	+	+	+	+	-
	37	2	-	+	+	+	+	+	+	-
	40	3	+	+	+	+	-	+	+	+
	32	3	-	-	+	-	+	+	+	+
	5	4	-	+	+	+	+	-	-	-
	54	4	-	+	-	+	-	-	-	-
CHA	39	3	+	+	+	+	+	+	+	-
	11	4	+	+	-	+	+	+	-	-
	59	4	-	+	-	+	-	-	-	-
	24	4	-	+	-	-	-	-	-	-

*(+) = Isolation of Nagasaki strain; (-) = Negative culture for Nagasaki strain

†Ns: nasal swab, Tr: traqueal swab, Pl: pleural swab, Lu: lung, Pc: pericardial swab, Lv: liver, Pt: peritoneal swab, Jt: joint swab.

Table 5.6. Number of pigs with positive cultures for *Haemophilus parasuis* Nagasaki strain from nasal or laryngeal swabs after inoculation with a low dose.

	Days of the study						
Groups	-3	2	3	5	7	18	Total[6]
EXP[1]	0/10	5/10	2/10	1/8[4]	3/8	0/8	8/10[a]
ABT[2]/EXP	0/10	1/10	1/10	4/10	8/10	0/8[5]	9/10 [a]
EXP/ABT[3]	0/10	2/10	5/10	0/10	0/10	0/10	5/10 [a]
ABT	0/10	0/10	0/10	0/10	0/10	0/10	0/10 [b]
CHA	0/10	0/10	0/10	0/10	0/10	0/10	0/10 [b]
NEG	0/10	0/10	0/10	0/10	0/10	0/10	0/10 [b]

[1] Low dose inoculation on day 0.

[2] Enrofloxacin treatment was given on day -3.

[3] Enrofloxacin treatment was given on day 3.

[4] Two pigs were euthanized on day 4 of the study.

[5] Two pigs were euthanized on day 7 and 16 of the study.

[6]Total number of pigs with at least one positive culture for Nagasaki strain.

Different letters indicate significant differences between groups ($p<0.05$).

Table 5.7. Number of pigs with positive cultures for *Haemophilus parasuis* farm strain from nasal and laryngeal swabs.

	Days of the study						
Groups	-3	2	3	5	7	18	Total[e]
EXP[a]	8/10	3/10	3/10	4/8	1/8	3/8	10/10
ABT/EXP[b]	5/10	0/10	0/10	0/10	0/9	0/8	5/10
EXP/ABT[c]	6/10	6/10	1/10	0/10	0/10	0/10	10/10
ABT[b]	4/10	5/10	NS[d]	NS	0/10	0/10	5/10
CHA	4/10	3/10	NS	NS	6/10	5/10	9/10
NEG	5/10	6/10	NS	NS	3/10	6/10	9/10

[a] Low dose inoculation took place on day 0.

[b] Enrofloxacin treatment was given on day -3.

[c] Enrofloxacin treatment was given on day 3.

[d] Not sampled.

[e] Total number of pigs tested positive for the Nagasaki isolate.

Figure 5.1. Experimental design timeline.

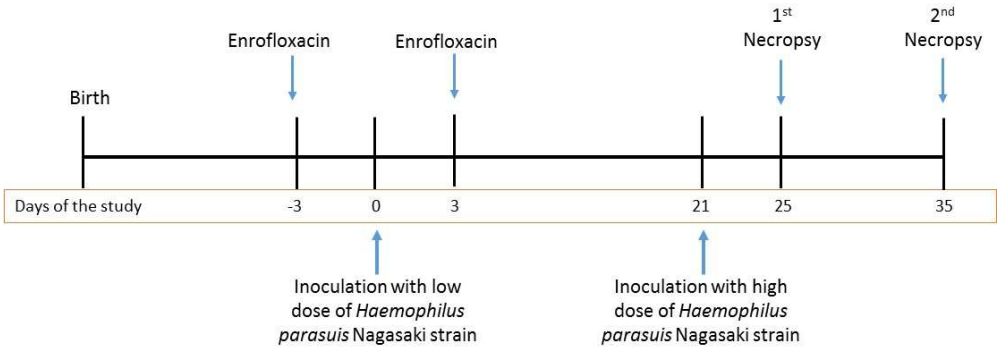


Figure 5.2. Average levels of serum IgG antibodies optical density (OD) prior to start the study (day -3), prior to challenge (day 17) and at necropsy (days 25 or 35). Different letters indicate significant differences between groups ($p<0.05$).

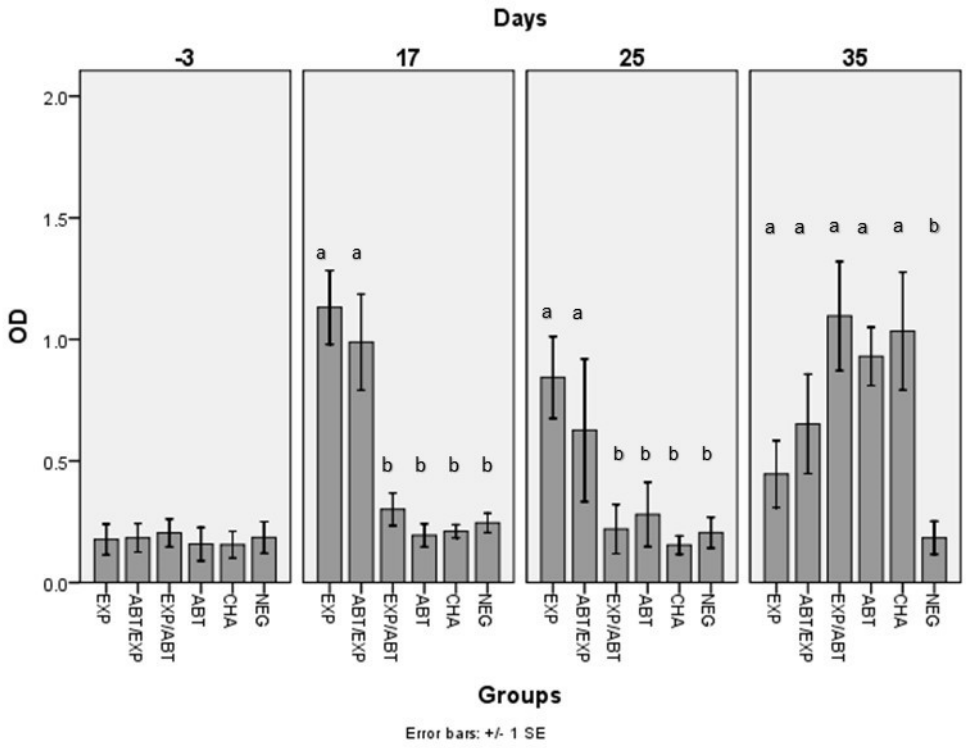


Figure 5.3. Average levels of IgA antibodies optical density (OD) in serum prior to start the study (day -3), prior to challenge (day 17) and at necropsy (days 25 or 35). Different letters indicate significant differences between groups ($p<0.05$).

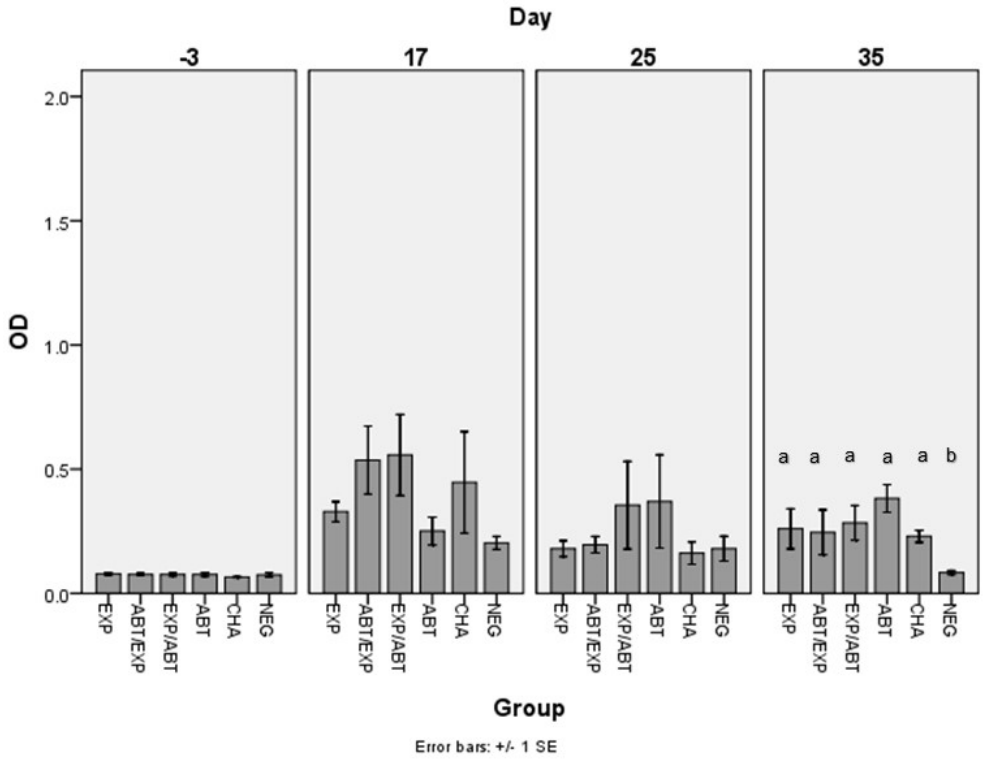


Figure 5.4. Average levels of IgG antibodies optical density (OD) against *Haemophilus parasuis* in BALF at necropsy (days 25 or 35). Different letters indicate significant differences between groups ($p<0.05$).

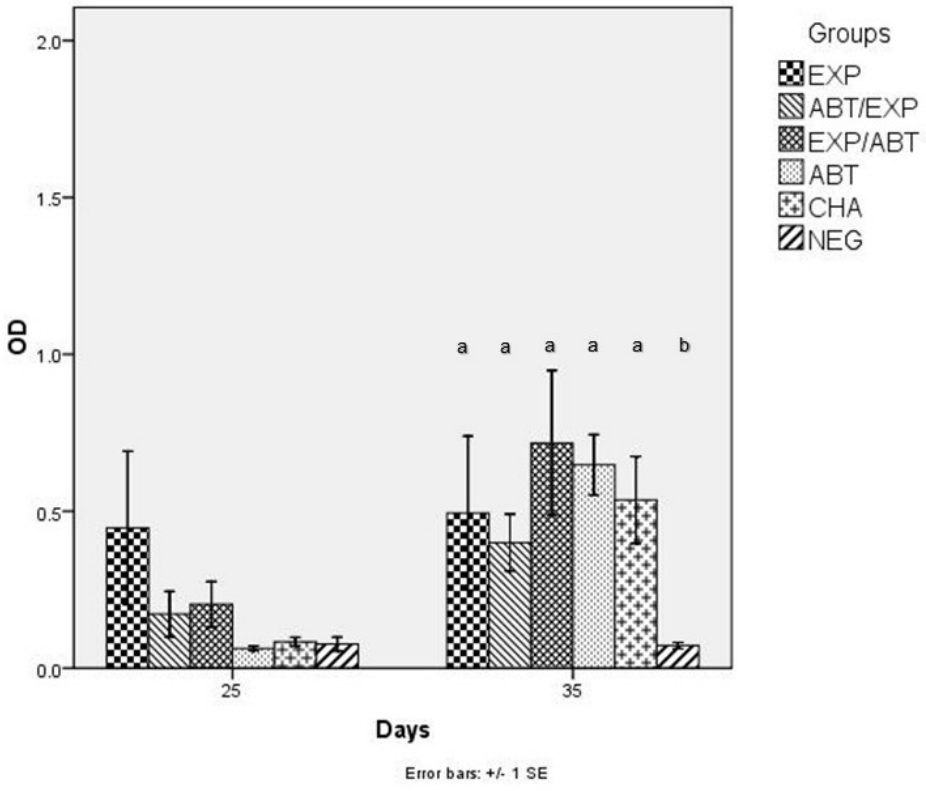


Figure 5.5. Average levels of IgA antibodies optical density (OD) against *Haemophilus parasuis* in BALF at necropsy (days 25 or 35). Different letters indicate significant differences between groups ($p<0.05$).

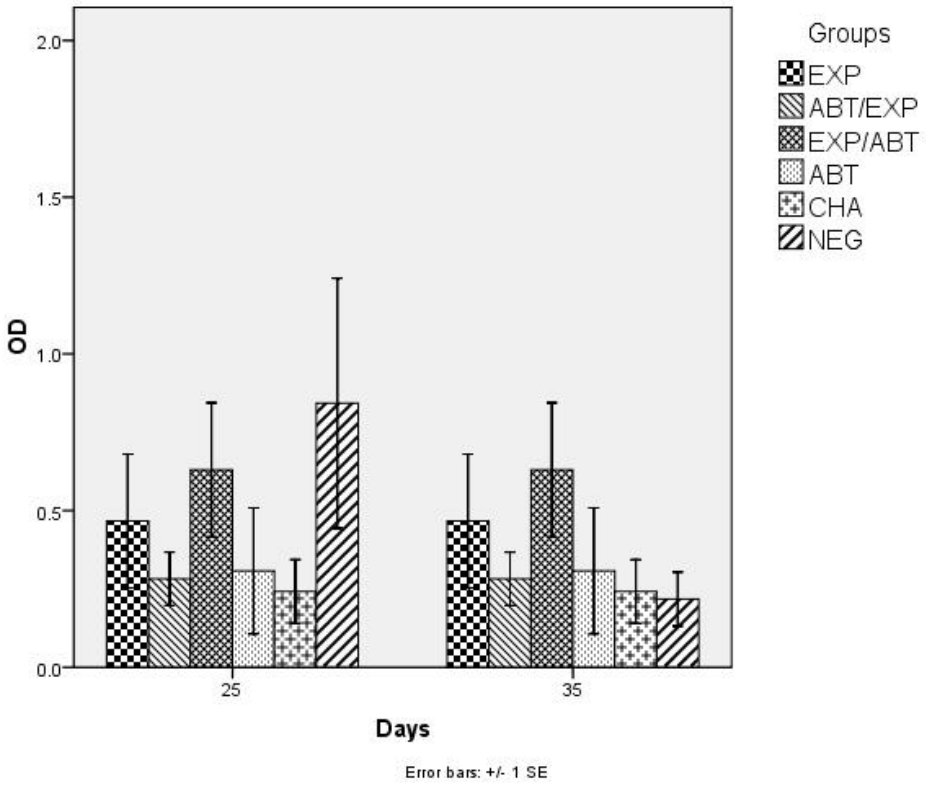
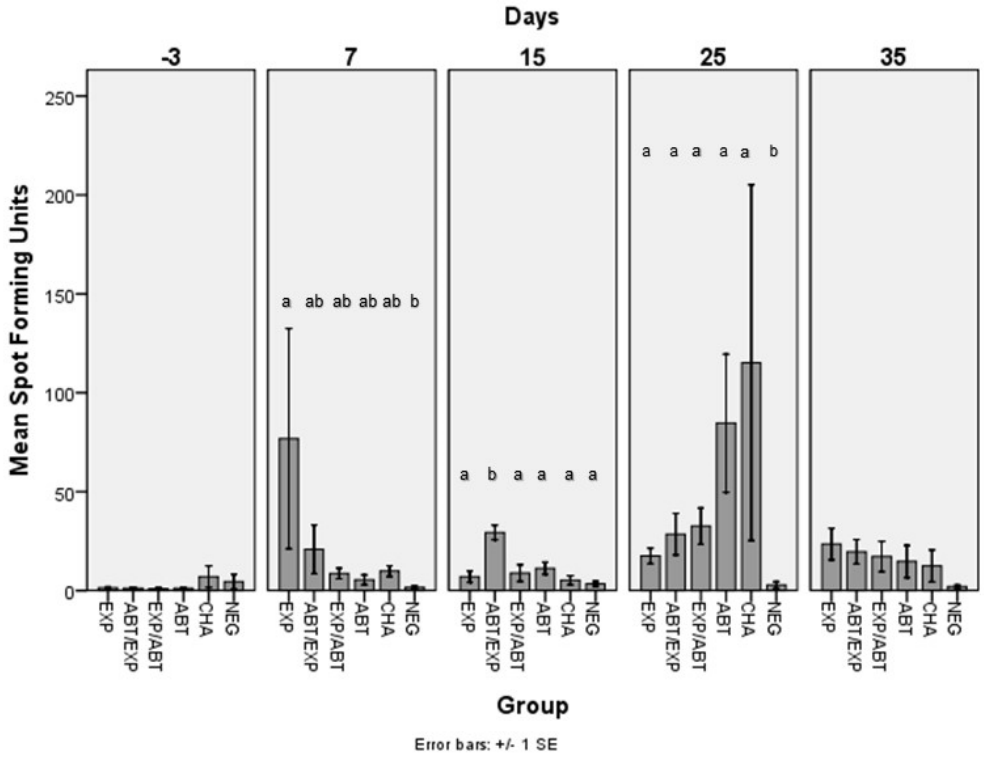


Figure 5.6. Average number of *Haemophilus parasuis*-specific interferon gamma (IFN-γ) spot forming units (SFU) per 10^6 cells by day Different letters indicate significant differences between groups ($p<0.05$).



CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

Glasser's disease is an important source of economic losses in commercial swine production systems. The success of controlled exposure in the field suggests that early exposure (colonization) with pathogenic *H. parasuis* protects pigs from Glasser's disease (Oliveira et al., 2004a). However, little is known about the immune response that is generated during colonization rather than during systemic infection. Understanding *H. parasuis* colonization and the associated immune response will contribute to the development of better control programs.

There is also a need to understand the factors that can modulate the immune response to *H. parasuis*, such as the use of antibiotic treatments. Over the past few years, use of antimicrobials has become more common in young pigs. Increase of *H. parasuis* disease has been observed in selected herds with an increase in use of antibiotics. It is possible that alteration of colonization patterns of *H. parasuis* at a young age has resulted in an increase in *H. parasuis* problems as the pigs get older. While the use of antibiotic medication helps pigs recover from *H. parasuis* infection, it may also affect *H. parasuis* colonization and alter the development of an effective immune response. Therefore, the effect of antibiotics on the development of an effective immune response requires further investigation. Towards this end, the goal of this PhD dissertation was to investigate the influence of antimicrobial treatment on *H. parasuis* colonization and infection, and its effect on the development of immune responses against *H. parasuis* in swine.

To investigate the serological immune response against *H. parasuis*, an ELISA assay was developed. The ELISA assay was based on a newly identified *H. parasuis*

antigen (chapter 2). The immunogenic and species-specific *H. parasuis* protein was detected by screening *H. parasuis* whole cell proteins using swine convalescent sera. This protein was identified as an oligopeptide permease A (OppA). This ELISA test proved to be valuable to detect specific antibodies against *H. parasuis* in infected or vaccinated animals. Therefore, the OppA ELISA provides a method for surveillance of *H. parasuis* infections. This test was useful for the subsequent chapters of this thesis and should be an important new tool for the study of *H. parasuis* pathogenesis and immune response in both experimental and field studies.

The immunogenicity of OppA was evidenced by the fact that only convalescent pigs or pigs vaccinated with recombinant OppA (rOppA) developed antibodies against it. Healthy pigs colonized by *H. parasuis* did not possess antibodies against OppA. The specificity of OppA was evidenced by absence of anti-OppA antibodies in colostrum-deprived pigs, SPF pigs and pigs infected with *A. pleuropneumoniae* or other common swine bacterial species. The robust antibody response to rOppA protein after vaccination further showed that OppA is highly immunogenic in swine. However, OppA protein seroconversion did not translate to protection against *H. parasuis* infection.

A major limitation of chapter 2 is that few serum samples of a limited number of farms were used to validate the OppA ELISA test. Future studies should use additional samples especially from pigs known to be free of *H. parasuis* and pigs experimentally inoculated with different *H. parasuis* strains. Evaluation of OppA ELISA under field conditions is also important. Specifically, the evaluation of antibody responses to OppA on farms with and without episodes of Glasser's disease

and, whether the OppA ELISA is a reliable tool to monitor the transfer of maternal immunity against *H. parasuis* to piglets need further investigation.

In chapter 3, the goal was to develop an experimental model of pathogenic *H. parasuis* that mimics asymptomatic colonization in conventional pigs. Such a model was necessary to study factors that affect colonization of *H. parasuis* in conventional pigs. In our model, pigs were experimentally inoculated with pathogenic *H. parasuis* and we showed that all pigs carried pathogenic *H. parasuis* on their URT mucosa for up to seven days post inoculation without developing Glasser's disease. Absence of disease was evidenced by lack of clinical signs and lesions, and lack of *H. parasuis* isolation and *H. parasuis* DNA detection in blood and systemic tissues. The colonization model developed as part of this thesis provides a new tool to study the pathogenesis and immune response to *H. parasuis* in conventional pigs.

While swine are the ideal model to study *H. parasuis* pathogenesis, there are negative aspects to using conventional swine. In general, pigs are naturally colonized with *H. parasuis* strains shortly after birth. The pigs used in this study were known to be *H. parasuis* positive and carried a genetically distinct strain of *H. parasuis* as part of the URT commensal bacteria. Although the commensal *H. parasuis* strain was identified and differentiated from the inoculated strain Nagasaki, it is possible that interactions between the 2 strains may exist. We did not account for possible interactions in this study as only pigs from a single source were used. Furthermore, we could not differentiate whether some of the responses observed were due to the presence of commensal strains, the pathogenic strain or the interaction among them. The *H.*

parasuis strain naturally established on the nasal mucosa is a complicating factor for the reproducibility of this model and further validation is needed.

In chapter 4, we evaluated the effect of the antimicrobial enrofloxacin on the colonization of *H. parasuis* in conventional pigs. We did this to evaluate whether enrofloxacin had an effect on *H. parasuis* found in the URT. We documented the reduction of the number of pigs testing positive to *H. parasuis* and the reduction of levels of *H. parasuis* in tonsils and nasal cavity of conventional pigs during the first week after enrofloxacin treatment. However, results also showed that enrofloxacin at the approved dose did not eliminate *H. parasuis* from nose and tonsils of the pigs. In this study, we could not differentiate between complete elimination and re-exposure from environmental sources, or presence of low grade persistence and recrudescence after the antibiotic effect was gone. This study was important to show that colonization of *H. parasuis* in healthy pigs can be altered by antimicrobial treatment and further supported the general hypothesis of this thesis. Prior to initiate this study, a minimum number of piglets was chosen based on estimated standard deviations and means, since a power analysis was not feasible. There was no preliminary data available to educate sample size selection, yet significant differences in number of pigs colonized by *H. parasuis* were detected.

The study in chapter 4 did not address whether the reduction on *H. parasuis* load by enrofloxacin can compromise the ability of pigs to develop an immune response when pigs are infected with a pathogenic *H. parasuis* strain. The study in chapter 5 was conducted to determine the effect of enrofloxacin on the development of immune responses to *H. parasuis* and its impact on protection from challenge. The inoculation

of pigs with a low dose of pathogenic *H. parasuis* protected pigs against challenge. In addition, we showed that timing of enrofloxacin administration in relation to *H. parasuis* exposure was important to develop protection to a subsequent *H. parasuis* challenge. The protection observed after *H. parasuis* inoculation was not affected by enrofloxacin given 3 days before inoculation. In contrast, enrofloxacin given 3 days after inoculation with *H. parasuis* interfered with protection against challenge. Based on the lack of *H. parasuis* isolation after enrofloxacin treatment, it appeared that the antibiotic quickly inactivated and removed *H. parasuis*. Alternatively, fluoroquinolones are known to have modulating effects on the immune response, by decreasing the synthesis of pro-inflammatory cytokines, such as IL-1 and TNF (Khan et al., 2000), which could have contributed to the absence of immune response. More research is needed, however, to investigate how enrofloxacin would affect the immune response to *H. parasuis.*

The serological assay used to measure IgG antibodies in this study was the OppA ELISA described in chapter 2. In chapter 5, we showed that pigs protected against challenge had an increase of OppA antibodies in serum after inoculation with a low dose of *H. parasuis*. In this study, anti-OppA antibodies were the main immune correlate of protection from Glasser's disease. In contrast, no differences were observed on levels of serum IgA antibodies and levels of IgG and IgA in BALF between inoculated groups. The IFN-γ response appeared to be associated with the onset of clinical disease after *H. parasuis* inoculation, but a clear association of IFN-γ response to protection was not evident. Therefore, more research is needed to evaluate the role of IFN-γ responses on protection against *H. parasuis* infection.

Results from chapter 5 also suggest that the use of potent antimicrobials, such as enrofloxacin, to control bacterial disease before pigs have time to activate a protective immune response may result in clinical disease when pigs become reinfected. On the other hand, treating pigs when clinical signs of disease are evident does not seem to interfere with protection against re-infection because the immune response may already have been activated. Overall more work to understand the immune response against *H. parasuis* infection is needed to evaluate the protective immune mechanisms against *H. parasuis* infection.

Taken together, the information generated in this thesis provides essential information on proper timing of antimicrobial treatment in the face of *H. parasuis* infection and immune response development. Data presented here showed that, for the given infectious dose of pathogenic *H. parasuis* used, more than 3 days of live bacteria exposure is necessary for pigs to seroconvert and be protected against reinfection. Enrofloxacin-induced abridgement of infection on day 3 post inoculation resulted in the absence of serum IgG response and protection.

The conclusions drawn from the data presented in chapter 5 may not be extendable to other pathogens or antimicrobials. Future studies should focus on investigating whether these conditions would change based on dose of *H. parasuis* inoculum, time of antimicrobial treatment and *H. parasuis* strains used. A complementary line of research would focus on the field application of the concepts presented in this thesis. Clinical trials can be developed that use the data provided in this thesis to compare different protocols of enrofloxacin administration in the face of a Glasser's disease outbreak.

Moreover, new efforts to understand the mucosal immune response to *H. parasuis* are needed, since serum IgA results in this study were inconclusive. The fact that secreted antibodies in the URT were not measured in piglets is a limitation since the pigs were naturally colonized. Future studies need to differentiate immune responses to the naturally colonizing farm strains from the immune responses to the inoculated strains to clarify how the pig's immune system responds to *H. parasuis*.

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